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# Neuroprotective Molecules and Strategies in a Rat Model of Neonatal Hypoxic Ischemic Encephalopathy

Brandon Joseph Dixon

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LOMA LINDA UNIVERSITY  
School of Medicine  
in conjunction with the  
Faculty of Graduate Studies

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Neuroprotective Molecules and Strategies in a Rat Model of Neonatal  
Hypoxic Ischemic Encephalopathy

by

Brandon Joseph Dixon

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A Dissertation submitted in partial satisfaction of  
the requirements for the degree  
Doctor of Philosophy in Physiology

---

December 2016

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Each person whose signature appears below certifies that this dissertation in his/her opinion is adequate, in scope and quality, as a dissertation for the degree Doctor of Philosophy.

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## ACKNOWLEDGEMENTS

I would like to express my deepest gratitude to Dr. Jiping Tang and Dr. John H. Zhang for providing examples of the work ethic of very successful scientists. As well as providing many opportunities for grantsmanship, reviewing articles, and publishing. I would also like to thank you both for all of your time, money, energy, passion, and sacrifice that you have invested in me. I have learned a tremendous amount of professional lessons from you both and hope that you can be proud of the scientist you have fashioned with your efforts.

I would also like to thank my committee members Dr. Ashwal, Dr. Gridley, and Dr. Pearce for their advice and direction. Dr. Ashwal and Dr. Gridley thank you for always making time for my presentation updates and sharing ideas with me. To Dr. William Pearce, thank you for the recommendation of using the Li-Cor and fluorescent antibodies as well as providing tips on how to use various computer software settings. To those in the Zhang Neuroscience Laboratory and the Center for Stroke Research with whom I have the pleasure of collaborating thank you for your time, support, and efforts. Special thanks to my HI group members throughout the years especially Jay Malaguit and Desi Doycheva.

To my friends at the Center for Health Disparities and Molecular Medicine who have become my family thank you for the support throughout this part of my journey. Special thanks to Dr. Johnny Figueroa, and Drs. Daisy and Marino De Leon and all the administrators and members of the IMSD program who have provided tremendous help and support throughout this endeavor. I would like to thank God for providing for me each step of the way and the opportunity to study His creation.

## DEDICATION

I would like to dedicate this dissertation to my immediate family, specifically my grandmother Dr. Inez Dixon. Thank you for providing countless examples of dedicating our lives to serving others, education, family, and trusting God. Also thank you grandmother Mary Kate McNeil for your constant encouragement, prayers, and love. Thank you Ada Dixon (Mom) and Roy Dixon (Dad) for always believing in me and providing words of wisdom in difficult times, encouraging video, pictures, and texts. I would also like to thank my beautiful and loving wife, Nichelle, for always encouraging me to keep going, for having study “date nights” and for your endless love and support. Thank you all! I would not be able to accomplish this portion of my journey through life without you, your prayers, and God!

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## ABBREVIATIONS

HIE	Hypoxic Ischemic Encephalopathy
ATP	Adenosine Triphosphate
BBB	Blood-Brain Barrier
VEGF	Vascular Endothelial Growth Factor
MMP	Matrix Metalloproteinases
ROS	Reactive Oxygen Species
G-CSF	Granulocyte-Colony Stimulating Factor
PI3K	Phosphoinositide 3-Kinase
VCAM-1	Vascular Cell Adhesion Protein 1
ICAM-1	Intercellular Adhesion Molecule 1
tPA	Tissue Plasminogen Activator
MRI	Magnetic Resonance Imaging
SVZ	Subventricular Zone
BDNF	Brain-Derived Neurotrophic Factor
mTOR	Rapamycin
CNS	Central Nervous System
STEP	Safety And Toxicity For Pediatrics
ESNEE	European Study of Neonatal Excipient Exposure
OPN	Osteopontin
i.p.	Intraperitoneal
IFN $\beta$	Interferon Beta
MS	Multiple Sclerosis

TNF- $\alpha$	Tumor Necrosis Factor Alpha
Epo	Erythropoietin
CSF	Cerebral Spinal Fluid
STAT	Signal Transducer And Activator Of Transcription
ERK	Extracellular Signal-Regulated Kinase
TPM	Topiramate
MgSO <sub>4</sub>	Magnesium Sulfate
NMDA	N-methyl-d-aspartate
CB	Cord Blood
GABA	Glycine and $\gamma$ -aminobutyric Acid
MAC	Minimum Alveolar Concentration
LPS	Lipopolysaccharide
SAH	Subarachnoid Hemorrhage
MCAO	Middle Cerebral Artery Occlusion
HI	Neonatal Hypoxia Ischemia
MAPK	Mitogenactivated Protein
IP <sub>3</sub>	Inositol Triphosphate
Ca <sup>2+</sup>	Calcium
BCL-2	B-cell lymphoma-2
HIF-1 $\alpha$	Hypoxia Inducible Factor -1 $\alpha$
iNOS	Inducible Nitric Oxide Synthase
S1P	Sphingosine-1- Kinase
SK	Sphingosine Kinase

iNOS	Inducible Nitric Oxide Synthase
PKC	Protein Kinase C
GSK-3 $\beta$	Glycogen Synthase Kinase-3
G-CSFR	Granulocyte-Colony Stimulating Factor Receptor
JAK	Janus Kinase
DMSO	Dimethyl Sulfoxide
EB	Evan's Blue
IgG	Immunoglobulin G
RIPA	Radioimmunoprecipitation Assay Buffer
MPO	Myeloperoxidase
ICV	Intracerebroventricular
nHI	Neonatal Hypoxic-Ischemic
iN	Intranasal
FAK	Focal Adhesion Kinase



## ABSTRACT OF THE DISSERTATION

### Neuroprotective Molecules and Strategies in a Rat Model of Neonatal Hypoxic Ischemic Encephalopathy

by

Brandon J Dixon

Doctor of Philosophy, Graduate Program in Physiology

Loma Linda University, December 2016

Dr. Jiping Tang, Chairperson

Neonatal hypoxic ischemic encephalopathy (HIE) is a devastating disease that primarily causes neuronal and white matter injury and is among the leading cause of death among infants. Currently there are no well-established treatments; thus, it is important to understand the pathophysiology of the disease and elucidate complications that are creating a gap between basic science and clinical translation. In the development of neuroprotective strategies and translation of experimental results in HIE, there are many limitations and challenges to master based on an appropriate study design, drug delivery properties, dosage, and use in neonates. Since there are no established therapies for HIE and it is critical to develop treatments that provide protection after HIE. In three separate studies we evaluated the efficacy of interferon beta (IFN $\beta$ ), granulocyte-colony stimulating factor (G-CSF), and osteopontin (OPN) in reducing apoptosis and inflammation following neonatal hypoxic ischemic encephalopathy and have outlined the cellular pathways involved in their abilities to provide neuroprotection. Our work showed that intranasal administration of IFN $\beta$  was able to be detected in the central nervous system, reduce brain infarction volumes, and improve neurological behavior tests 24 hours after HIE. Administration of G-CSF was able to play a pivotal role in attenuating

neuroinflammation and BBB disruption 48 hours following HIE. Similarly, OPN was able to decrease blood-brain barrier permeability and brain edema after HIE. In addition, we have also reviewed and discussed the current literature on the pathophysiology, potential intervention sites, novel neuroprotective molecules, clinical trials, and future directions concerning HIE.

**CHAPTER ONE**

**NEUROPROTECTIVE STRATEGIES AFTER NEONATAL HYPOXIC  
ISCHEMIC ENCEPHALOPATHY**

**Abstract**

Neonatal hypoxic ischemic encephalopathy (HIE) is a devastating disease that primarily causes neuronal and white matter injury and is among the leading cause of death among infants. Currently there are no well-established treatments; thus, it is important to understand the pathophysiology of the disease and elucidate complications that are creating a gap between basic science and clinical translation. In the development of neuroprotective strategies and translation of experimental results in HIE, there are many limitations and challenges to master based on an appropriate study design, drug delivery properties, dosage, and use in neonates. We will identify understudied targets after HIE, as well as neuroprotective molecules that bring hope to future treatments such as melatonin, topiramate, xenon, interferon-beta, stem cell transplantation. This review will also discuss some of the most recent trials being conducted in the clinical setting and evaluate what directions are needed in the future.

**Introduction**

***Neonatal Hypoxic Ischemic Encephalopathy***

Neonatal hypoxic ischemic encephalopathy (HIE) is a devastating disease that primarily causes neuronal and white matter injury. HIE has tremendous detrimental effects on the developing brain and is among the leading causes of death among infants, as well as the major underlying cause of seizures in term infants [1,2,3]. Although there

have been major advances in modern technology and an increased understanding of fetal and neonatal pathologies, HIE is still a serious condition that is unresolved and causes significant mortality and long-term morbidity [4,5,6,7].

Neonatal HIE can also be characterized as an injury that occurs in the immature brain, resulting in delayed cell death via excitotoxicity, inflammation, and oxidative stress [4]. These adverse events in the developing brain often lead to long lasting detrimental neurological defects later on in life such as mental retardation, epilepsy, cerebral palsy, learning disabilities, and other neurophysiological handicaps [8]. Care for newborn infants at risk for hypoxia ischemia is a priority in health care and understanding the pathophysiology of hypoxic ischemic brain injury is quite essential to the design of effective interventions [9].

Before the advent of hypothermia, clinicians were not able to provide much care to neonates suffering from HIE besides systemic supportive care [10]. It is necessary to explicate interventions that will rid young children's lives of this form of stroke. Thus, this review aims to characterize the current pathophysiology of HIE, and describe and elucidate complications that are creating a gap between basic science and clinical translation. In addition, we aim to analyze promising neuroprotective strategies after HIE and prognosticate how treatment strategies will change and what new therapeutic strategies are on the horizon.

### ***Incidence and Prognosis of HIE***

Neonatal hypoxic ischemic encephalopathy is of great importance since it is the major cause and contributor to global infant mortality and morbidity [11]. The incidence

of neonatal HIE in the United States is 2–3 in 1000 live births, with evidence of incidences being up to 6 births in 1000 live births [11]. Underdeveloped countries have even reported incidences up to 26 per 1000 live births [10]. About 20%–25% of term newborn infants die during the neonatal period and about 25% of those that survive develop permanent neurological disabilities. Patients with mild grades of encephalopathy are generally reported to have normal cognitive functions by school age. While patients with moderate grading are associated with a spectrum of long-term disabilities and significant motor and cognitive disabilities [10].

### ***Clinical Presentation***

Neonatal hypoxic ischemic encephalopathy usually presents clinically in the earliest days of life in a term infant and can be characterized by difficulty initiating and maintaining respiration, depression of tone and reflexes, subnormal levels of consciousness, and multiple seizures [10].

Since HIE is the major underlying cause of seizures in term infants, a large variety of seizures may present [12]. Subtle, clonic, tonic, myoclonic that are focal, multifocal, or generalized are the most common types of seizures that present [13,14,15]. In the perinatal period hypoxemia, ischemia, and other impairments to the exchange of respiratory gases often give rise to asphyxia. Thus, neonatal HIE is described as acute intrapartum events that cause moderate to severe neonatal encephalopathy, metabolic acidosis in fetal umbilical arterial blood obtained at delivery, spastic or dyskinetic quadraparesis, and absence of other causes of cerebral palsy [16]. It is also critically

important to eliminate other causes of neonatal encephalopathy in order to prevent delay in diagnosis and neuroprotective intervention [17].

In order to classify neonatal HIE and the degree of injury, the Sarnat 3 stage grading system is used and widely accepted. This system consists of three stages ranging from mild, moderate, and severe, all based on clinical symptoms described above, along with electroencephalogram evaluation [18].

### ***Current Therapeutic Strategies***

Presently, there are no well-established effective therapies for neonatal HIE [19]. Hypothermia is a method of protection that is used to treat full term neonates with moderate to severe HIE. Along with hypothermia, comprehensive clinical care of mechanical ventilation, physiological and biochemical monitoring, neuroimaging, seizure detection and monitoring, and neurological consultation are also included. Although hypothermia does provide some protection, only 1 in 6 infants benefit from hypothermia [20]. There is an area of uncertainty involving hypothermia in regards to depth and duration of cooling, and information involving premature infants born less than 35 weeks of gestational age. Thus, it is critical to have an appropriate treatment to provide protection to neonates suffering from HIE [21].

### **Potential Intervention Targets**

Since the pathophysiology of HIE injury is quite complex, there are a myriad of potential interventional targets following HIE where prevention of cellular damage can occur [22] (Figure 1). Targeting impaired function sites like the neurovascular unit,

attempting to quell apoptosis, inflammation, or promoting neurogenesis and angiogenesis are all strategic points of importance [10,23]. Although targets such as apoptosis or necrosis have been previously explored, there are other areas of importance that have been understudied [24,25]. We will now delve into the pathophysiology and discuss some promising understudied intervention sites.

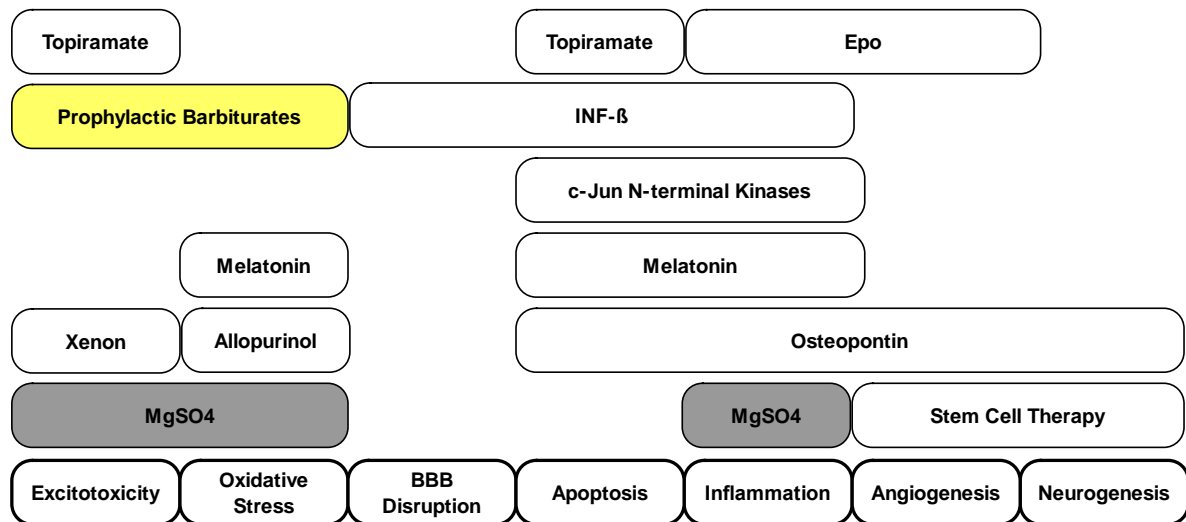


Figure 1. Interventional Targets Following Hypoxic Ischemic Encephalopathy (HIE). A summary of potential interventional targets following HIE along with molecules that can exert multiple properties. Interventional targets consist of Excitotoxicity, Oxidative Stress, Blood Brain Barrier Disruption, Apoptosis, Inflammation, Angiogenesis, and Neurogenesis. Multiple target sites suggest that a multi-targeted approach is beneficial after HIE.



### ***Pathophysiology***

The next phase that follows is a primary energy failure phase that occurs at the cellular level (Figure 2a). Since there is a loss of oxygen that is readily available to the brain, cellular energy metabolism shifts to a dependency upon anaerobic metabolism. This reliance upon anaerobic metabolism pathways leads to the collection of lactic acid and depletion of adenosine triphosphate (ATP) [27]. The loss of cellular homeostasis also leads to an intracellular accumulation of sodium, calcium, water, and excitatory neurotransmitter release causing an “excitotoxic-oxidative cascade”. Increasing cellular influx of calcium also occurs as a consequence of excessive stimulation of neurotransmitter receptors and membrane depolarization [28,29].

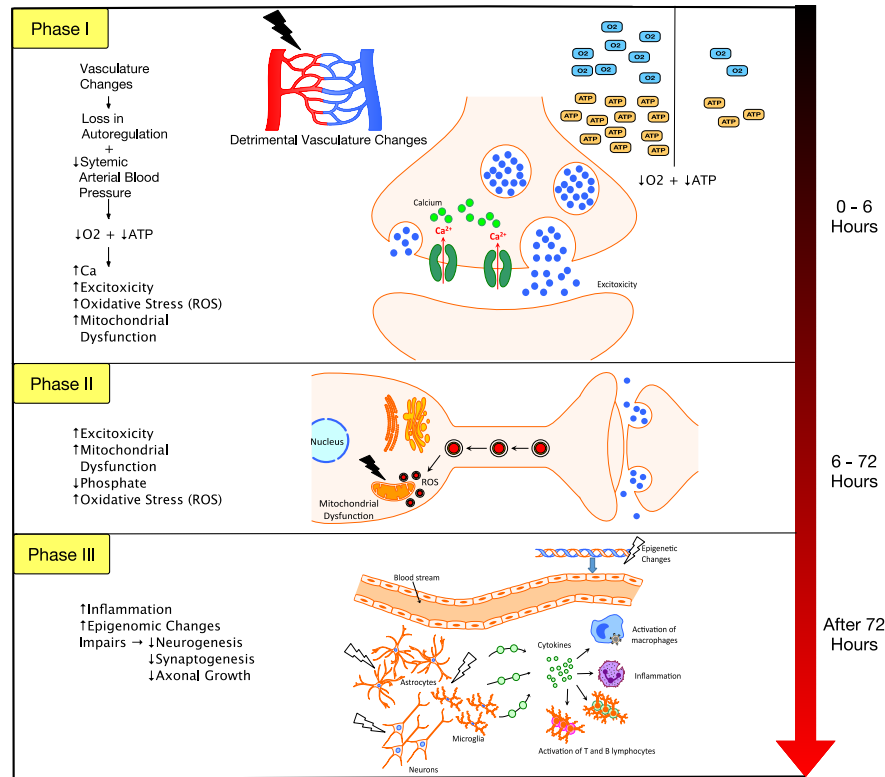


Figure 2. Vasculature Changes and Primary Energy Failure (Phase I) Legend: A visual representation of the first phase of HIE. Detrimental changes to the vasculature following an HIE insult lead to loss of autoregulation and severe lowering of the systemic arterial blood pressure. This causes a decrease in oxygen, depletion of ATP, as well as increases in excitotoxicity, intracellular calcium, oxidative stress, and mitochondrial dysfunction; (b) Secondary Energy Failure (Phase II). Legend: A schematic representation of the second phase of HIE reveals continued excitotoxicity, oxidative stress, and mitochondrial dysfunction; (c) Chronic Inflammation (Phase III). A pictorial representation of the third phase of HIE shows injury to microglia, neurons, and astrocytes leads to continuous release of cytokines and other detrimental factors causing chronic inflammation which in turn leads to epigenetic changes, as well as impairments of synaptogenesis, axonal growth, and neurogenesis.

Further influx of calcium also leads to increased activation of lipase, causing a release of fatty acids, and increased activation of neuronal nitric oxide synthase giving rise to free radical production and mitochondrial dysfunction [26]. As a consequence, mitochondrial dysfunction ultimately signals pathways of apoptotic or necrotic cell death. Apoptotic cell death is believed to occur when energy supplies are not completely exhausted, while necrotic cell death occurs when energy supplies are no longer available [27].

A second energy failure phase also occurs 6–48 h after an episode of hypoxia ischemia [30]. The second energy failure phase also results in the detrimental release of excitatory neurotransmitters and free radicals as well as depletion of high phosphate reserves, but differs from the primary energy failure phase since it is independent of cerebral acidosis [30,31].

A third phase where deleterious factors cause further damage and potentiates injury and worsens outcomes has recently been proposed. This third phase is thought to include mechanisms of inflammation and epigenetic changes that lead to an impairment or alteration of axonal growth, neurogenesis, and synaptogenesis [29].

### ***Blood-Brain Barrier***

The neurovascular unit, also referred to as the blood-brain barrier (BBB), consists of the basement membrane, capillary endothelial cells, tight junctions, pericytes, and astrocytes [32]. It is believed that all the components that comprise the BBB are important for stability and proper functioning [33]. Thus, injury to the cerebrovasculature and the BBB after HIE leads to detrimental effects since it is an essential diffusion barrier

required for normal functioning of the central nervous system [33]. In an observational study of term neonates with HIE, it was found that BBB permeability is a contributing factor to HIE [34]. In order to assess permeability of the BBB in this study, the cerebrospinal fluid albumin to plasma albumin ratio was measured in 43 HIE neonates and was compared with 20 normal gestational age and gender-matched healthy infants without HIE. The results showed that plasma albumin levels were similar in neonates with and without HIE. However, cerebral spinal fluid (CSF) albumin levels were 5 times higher in infants with HIE when compared with controls indicating permeability of the BBB [32,34,35].

It is believed that cytokines, nitric oxide, and vascular endothelial growth factor (VEGF) help regulate mechanisms that lead to tight junction disruption and increased BBB permeability [33]. Increased levels of proinflammatory cytokines have also been observed in animal brains after global and focal ischemia as well as in cerebrospinal fluid of stroke patients [33].

After HIE it is postulated that there are biphasic temporal patterns of BBB opening. Numerous in vitro and in vivo studies have revealed that VEGF and matrix metalloproteinases (MMP) are involved in the initial opening of the BBB within hours of an hypoxic-ischemic event [32]. While the second opening, occurring around 6–24 h, involves the activation of microglia and astrocytes which release proinflammatory cytokines that induces MMPs and cyclooxygenases triggering protease destruction and reactive oxygen species (ROS) production respectively [32]. Once the barrier is disrupted, detrimental events can be further exacerbated by the infiltration of neutrophils and monocytes from the systemic compartment.

Although many adverse outcomes occur once the BBB is permeable, there are experimental studies that show that stabilizing the BBB and reducing inflammatory markers after hypoxic ischemic events as well as HIE may be beneficial [36,37,38,39,40]. For example, a study exploring the mechanisms of granulocyte-colony stimulating factor (G-CSF) as a treatment after a neonatal rat model of HIE, demonstrated that G-CSF elicited BBB stabilization by G-CSF receptor stimulation and activation of the phosphoinositide 3-kinase (PI3K)/Akt pathway. The enhancement of the BBB occurred in endothelial cells through increases in tight junction proteins, claudin 3 and claudin 5, in addition to decreases in vascular cell adhesion protein 1 (VCam-1) and intercellular adhesion molecule 1 (ICam-1) adherens proteins [41].

Similarly another study utilizing a neonatal rat model administered plasminogen activator inhibitor-1 following an HIE insult. This group was able to demonstrate that inhibition of tissue plasminogen activator (tPA) was able to prevent MMP activation, prevent HIE-tPA induced opening of the BBB, and decrease tPA-converted plasmin activities as a protease that normally degrades the extracellular matrix and the BBB [42].

Some strides have been made, yet further investigations are needed to better understand BBB integrity after HIE [35]. It has been suggested that endothelial cells may be more susceptible to oxygen deprivation, while astrocytes and pericytes are tolerant of sole oxygen deprivation, indicating that a more complete understanding of the cellular relationship that comprises the BBB may be critical to formulating strategies of protection following hypoxic ischemic events [43]. Also, more recent knowledge seems to indicate that the BBB may be more stable during the developmental period than maturation [35]. Thus, any and all information regarding the mechanisms of BBB

formation and disruption will help in constructing strategies of BBB protection.

### *Angiogenesis*

Angiogenesis is characterized as the process involving the growth of new vessels that extend the existing blood circulation into avascular regions. The angiogenesis process consists of vascular basal lamina formation, proliferation and migration, and tubular formation of migrating cells. Some findings suggest that angiogenesis is activated after acute insult in the neonatal brain [44]. One study comparing asphyxiated newborns without brain injury, asphyxiated newborns with brain injury, and healthy newborns discovered that angiogenesis pathways maybe dysregulated after HIE. This dysregulation was evidenced by the observation that asphyxiated newborns with brain injury had decreased expression of insulin-growth factor binding protein-1, -4, and -6, which are anti-angiogenic proteins. While asphyxiated newborns that did not develop brain injury showed increases in fatty acid binding protein 4, glucose-6-phosphate isomerase, expression of neuropilin-1 (a vascular endothelial growth factor receptor-2 co-receptor), and receptor tyrosine-protein kinase erbB-3, which are proteins associated with endothelial cell survival, proliferation, and migration [45]. Thus, angiogenesis seems to be a viable target in newborns with HIE [44]. In the adult brain, angiogenesis is a key repair mechanism after a hypoxic ischemic event. However, several factors concerning angiogenesis remains to be elucidated after injury in term newborns [44]. Further studies to discover how angiogenesis can be activated to enhance brain repair after HIE in newborns are essential to creating viable strategies on how to improve functional and structural recovery after injury [44]. One study indicated that angiogenesis is activated

after HIE injury by observing hyperfusion measured by magnetic resonance imaging (MRI) during the first month [44]. It has also been shown that angiogenesis could be induced by the transplantation of CD34+ cells from umbilical cord blood and that this improvement is beneficial [46]. Together the data suggests that the neovascularization mechanisms are essential for survival and may be a potential therapeutic site after stroke.

### *Neurogenesis*

Neurogenesis is comprised of cell proliferation, migration and differentiation [47]. It is believed that neurogenesis continues even throughout adulthood. The site for neurogenesis occurs in the subventricular zone and subgranular layer of the hippocampal dentate gyrus, where the local environment tightly regulates neurogenesis. Although there is evidence that suggests that neurogenesis increases after injuries such as HIE, the endogenous repair mechanisms do not resolve the brain damage that occurs [48,49,50]. Ong et al. observed increases of doublecortin, a microtubule-associated protein only expressed in immature neurons, with immunostaining in the subventricular zone ipsilateral to HIE-induced lesioning one to three weeks post injury. However, after the fourth week there were no identifiable newly formed mature striatal neurons, suggesting that there was limited neurogenesis at this time period. These results also suggest depletion of the neuronal progenitor pool since they are more vulnerable to HIE than SVZ stem cells or that the environment is not conducive to the maturation and survival of newly formed neurons since there maybe a lack of trophic support [51]. Thus, aiding or boosting the endogenous repair mechanisms after brain injuries may restore function. Therefore, understanding the molecular and cellular mechanism of neurogenesis

and whether or not these mechanisms can be clinically applicable presents neurogenesis as a prime therapeutic target [50,51].

There have been a number of promising stem cell therapy experiments that promote neurogenesis after injury. For example, van Velthoven et al. demonstrated that bone marrow derived mesenchymal stem cells treatment after HIE was able to increase neurogenesis and formation of new oligodendrocytes, evidenced by NeuN-positive and olig2-positive cells [52]. Again, it is quite important that more studies are conducted to optimize and elucidate mechanisms following injury. Discovering and understanding the factors that promote neurogenesis during normal physiological conditions as well as the impairment that occurs during brain damage are key questions that need to be determined [50,53].

### *Autophagy*

After HIE, there is evidence of differential cellular death mechanisms often occurring in different cells. One cellular death mechanism that has increased scientific interests and has been recently implicated in HIE has a potential therapeutic target is autophagy [24,54]. Autophagy is a lysosomal pathway for intracellular degradation of macromolecules and organelles that plays an important part to maintaining cellular survival and homeostasis [55]. Since autophagy is a tightly regulated process, controversy of whether or not autophagy provides beneficial effects after HIE currently exists [56]. There are some experiments that describe pharmacological inhibition of autophagy being neuroprotective, while others have shown that inducing autophagy immediately after injury may be an endogenous neuroprotective mechanism [4].



For example, Chen et al. demonstrated that brain-derived neurotrophic factor (BDNF) with a concentration range of 50 to 200 ng/mL protects neurons from hypoxia injury in vitro via the induction of autophagy [57]. It was shown that BDNF could induce autophagy through the PI3K/Akt/mammalian target of rapamycin (mTOR) pathway. In this study, activation of mTOR complex 1 lead to phosphorylation of ribosomal protein S6 kinase (p70S6K), a controller of protein translation, which ultimately induced autophagy and exerted protective effects [57]. Conversely, a study evaluating the effects of lithium in an in vivo model of neonatal HIE brain injury found that lithium was able to inhibit post-ischemic autophagy after 72 h. This reduction of autophagy was indicated by decreased number of LC3-positive cells in lithium-treated animals 72 h post-HIE. Given these results, it is unclear if a reduction of autophagy occurred as a secondary effect to lithium induced neuroprotection since there is less cellular debris and damage contributing to autophagy processing or if lithium directly inhibited autophagy [55]. Mixed results involving autophagy suggests that deleterious or neuroprotective effects depend upon specific regions, the severity of the insult, and the timing of activation [4]. Over the past decades, much has been learned about autophagy; however, more studies to elucidate exact mechanisms remain, especially following neurodegenerative insults and diseases [54].

### **Potential Novel Molecules and Strategies for Neuroprotection after HIE**

Over the past decade, clinical and basic science research in neonates has achieved huge progress in neuroprotective and neurointensive care, and established hypothermia as the standard of care treatment for neonatal HIE. Although there has been much progress,

further investigation is needed to discover adjuvant neuroprotective strategies [58]. This is also important for designing clinical and experimental studies since therapeutic targets might alternate in the phases of HIE, in addition to the physiological development changes that occurs in neonates over time [29]. We will now discuss some molecules that are neuroprotective and are currently being evaluated in experimental translational studies (Table 1) and clinical trials (Table 2) for therapeutic potential after HIE.

Table 1. Neuroprotective molecules evaluated in experimental translational studies after HIE. Summary of neuroprotective molecules used in experimental translational studies and their proposed mechanisms of action associated with HIE.

<b>Molecules Studied</b>	<b>Possible Effects Related to Neuroprotection</b>
Osteopontin (OPN)	OPN repairs brain injury after neonatal HIE by mediating regulation of cerebral cell proliferation, cell survival, and oligodendrocyte differentiation after injury [59]
Interferon Beta (INF $\beta$ )	Reduce TNF- $\alpha$ levels, proliferation and activation of T-cell lymphocytes, and pro-inflammatory cytokines produced by T-cells; Blood Brain Barrier integrity [60]
c-Jun N-terminal kinases (JNKs)	JNKs play a role in regulation of apoptosis [61]; Reductions in early neuronal damage [62]; Reduced inflammation and inhibition of apoptotic neuronal loss [63]
Prophylactic barbiturates	Diminishes moderate to severe neurodevelopmental impairment or death (HIE undergoing whole-body cooling) [64]; Multivariate analysis suggested its use to be associated with better outcomes [64]
Melatonin	Antioxidant, anti-inflammatory, and anti-apoptotic properties [65,66]; Protect the brain independently or in concert with therapeutic hypothermia [67]; Reducing oxidative stress and improved survival with favorable neurodevelopmental outcome at 6 months of age in combination with hypothermia [68]
Edaravone	Edaravone may inhibit the number of apoptotic neuronal cells and 8-OHdG expression within 48 h after HI insult [69]; Inhibits lipid peroxidation in neonatal HIE rat model [70]; Scavenger that inhibits both lipid and DNA peroxidation [71]

Table 2. Neuroprotective molecules evaluated in current clinical trials involving HIE. Summary of neuroprotective molecules that are currently being studied in HIE clinical trials and their proposed mechanisms of action.

Molecules Studied	Possible Effects Related to Neuroprotection
Erythropoietin (EPO)	Associated with anti-inflammatory, anti-excitotoxic, anti-oxidative, and anti-apoptotic properties [72,73,74]; Vasogenic and pro-angiogenic functions [73]; Hypoxia-inducible-factor-1 mediates increase in EPO expression [75,76]
Allopurinol	Neuroprotection in postnatal day 7 rats after HI [77]; Post hoc analysis revealed a potential benefit in treatment of females [78].
Xenon	May trigger neurodegeneration in the developing brain. Thus, the safety of a newborn injured brain is not expected [79]; Neuroprotective in adult rats in transient brain ischemia [79]; Limited protection when given alone but protection for up to 30 days when given in combination with hypothermia (neonatal rodents) [80]
Prophylactic barbiturates	Diminishes moderate to severe neurodevelopmental impairment or death (HIE undergoing whole-body cooling) [64]; Multivariate analysis suggested its use to be associated with better outcomes [64]
Topiramate (TPM)	AMPA and Kainate receptors inhibition [81,82,83]; Blockade of Na channels, high voltage-activated calcium currents, carbonic anhydrase isoenzymes and mitochondrial permeability transition pore [84,85,86,87]; TPM in concert with melatonin decreases infarcted volume and apoptosis in neonatal HI rat model [88]; Pretreatment significantly reduced the brain damage and subsequent cognitive impairments [89]
Magnesium Sulfate (MgSO <sub>4</sub> )	Controversies exist regarding its efficacy in protecting the brain in term infants who may suffer encephalopathy [90]
Cord blood	Controversies exist regarding its efficacy in protecting the brain in term infants who may suffer encephalopathy [91]

## *Experimental Translational Studies*

### **Osteopontin**

Osteopontin (OPN) is a multifunctional glycoprotein with increased upregulation in the brain after neonatal HIE. OPN has both pro- and anti-inflammatory properties; thus, its exact role in injury is not well predicted [59,98,99]. OPN repairs brain injury after neonatal HIE by mediating regulation of cerebral cell proliferation, cell survival, and oligodendrocyte differentiation after injury [100]. According to Chen and colleagues, OPN-induced neuroprotection was associated with cleaved caspase-3 inhibition and anti-apoptotic cell death, thereby improving long-term neurological function against neonatal HIE brain injury [101]. However, a recent study done by Bonestroo and colleagues demonstrated that intranasal, intraperitoneal (i.p.), and intracerebral administration of a small TAT-OPN peptide was neither neuroprotective by measuring the anatomical reduction of the HIE induced brain injury, nor beneficial in reducing sensorimotor behavioral deficits [102]. Endogenous expression of OPN was shown to be highest in the brain at age 0 with continuous reductions until day 21 during development. After HIE injury, endogenous OPN expression increased and peaked at 48 h. Exogenous OPN decreased infarct volume and improved neurological outcomes 7 weeks after HIE injury [101].

### **Interferon Beta**

Inflammation plays an important role in the pathology of HIE, interventions at the inflammation portion of the disease can potentially be beneficial [19]. Thus, it is plausible that the positive immunomodulatory effects of interferon beta (IFN $\beta$ ) as seen in

an inflammatory environment such as multiple sclerosis (MS) will also have a therapeutic effect in the neonatal HIE model. In experimental models of MS, IFN $\beta$  has been shown to reduce tumor necrosis factor alpha (TNF- $\alpha$ ) levels, proliferation and activation of T-cell lymphocytes, and pro-inflammatory cytokines produced by T-cells [60]. Intrastriatal injections IFN $\beta$  has been shown to preserve the BBB integrity, decrease infarct size, and block the infiltration of inflammatory cells in a middle cerebral artery occlusion model [103]. In a model of transient focal stroke, it was reported that intravenous tail injections of IFN $\beta$  failed to provide protection. It appears that IFN $\beta$  is unable to cross BBB; thus, methods to circumvent the BBB are needed in order for IFN $\beta$  to be effective [104].

### **c-Jun N-Terminal Kinases**

c-Jun N-terminal kinases (JNKs) activation is associated with an assortment of environmental stressors and for that reason they are known as stress activated protein kinases [105,106]. Through phosphorylation and modification of proteins residing in the mitochondria, JNKs play a role in regulation of apoptosis [61]. Nijboer and colleagues demonstrated reductions in early neuronal damage in P7 rats at 0 and 3 h after HIE [62]. They injected intraperitoneally TAT-JBD, a JNK inhibitor, in a neonatal model of HIE brain injury. Post insult administration reduced brain damage and lasted up to 14 weeks post-HIE. Furthermore, sensory, cognitive, and behavioral benefits were associated with the 50% anatomical cerebral improvements found in their study. These results indicated that the activity of JNK in the brain was inhibited effectively by TAT-JBD treatment [107]. In 2013 Nijboer and colleagues also demonstrated that inhibition of phosphorylation of mitochondrial JNK may lead to preventing early loss of mitochondrial

integrity, consequently leading to reduced inflammation and inhibition of apoptotic neuronal loss. Up-regulation of anti-apoptotic mitochondrial proteins also played a crucial role in maintaining neuroprotection [63].

The TAT-JBD peptide may serve as a treatment option for neonatal HIE due to its promising results in reducing neuronal damage and loss of mitochondrial activity through early JNK inhibition, with overall improvements in anatomical outcomes, therefore improving cognitive and behavioral results post-HIE. The present study shows that early JNK inhibition by the short-lived TAT-JBD peptide may be a promising therapy for neonatal HIE by conferring long-term anatomical and behavioral improvements [107].

### **Prophylactic Barbiturates**

A retrospective study by Donald F. Meyn, Jr. and colleagues [64] analyzed the effects of prophylactic administration of phenobarbital to infants with HIE. They found that phenobarbital administration to infants with HIE undergoing whole-body cooling diminishes moderate to severe neurodevelopmental impairment or death. Despite their small sample size, they found that this combination diminishes clinically detectable seizures. On the other hand, their study failed to improve neurodevelopmental outcomes significantly by univariate analysis. However, multivariate analysis suggested its use to be associated with better outcomes. Though the most effective dose, most effective timing of administration, and the most effective drug are not known, the treatment combination used by Meyn Jr. and colleagues may help disrupt the cascade of injury.

Therapeutic interventions enabling prevention or reduction in hypoxia-induced brain damage before or during an earlier stage of free-radical production will require

continued investigation for optimal effectiveness [108]. These results and findings set the stage for a large and multicenter randomized-control trial that includes a long term follow up analysis to further test the incremental benefit of prophylactic anticonvulsant therapy in the setting of hypothermia [64].

## **Melatonin**

Melatonin (N-acetyl-5-methoxytryptamine) is an endogenous indolamine and another scavenger that has shown promising effects in the treatment of HIE. It has antioxidant, anti-inflammatory, and anti-apoptotic properties [65]. Melatonin freely crosses the placenta and the blood-brain barrier making it an attractive agent for neuroprotection. In an asphyxia animal model it has been shown to protect the brain independently [66] or in concert with therapeutic hypothermia [67]. Aly and colleagues demonstrated that the combination of melatonin and therapeutic hypothermia in infants with moderate to severe HIE was efficacious in reducing oxidative stress and improved survival with favorable neurodevelopmental outcome at 6 months of age [68]. Intravenous use of melatonin showed efficacy and feasibility when used in neonates with HIE who were receiving whole body therapeutic hypothermia [68]. With its effectiveness for both pre-term and term infants [109] it holds considerable promise as an adjunct therapy [110] and results from various studies suggest combination therapy as the most effective. Optimal dose, route, and duration of administration are still parameters that need to be researched in depth in order to help in clinical translation.



## **Edaravone**

Edaravone (3-methyl-1-phenyl-2-pyrazolin-5-one) is a free scavenger that is thought to be useful for the treatment of acute cerebral stroke. Edaravone is believed to interact with peroxy and hydroxyl radicals creating a radical intermediate that forms stable oxidation products [69,70]. Ni X. and colleagues also demonstrated the benefits of edaravone as an antioxidant agent in HIE. They observed that systemic administration of edaravone 30 min after resuscitation from HIE can salvage neurons in the striatum in a large animal model of neonatal HIE [71]. Furthermore another study showed that intraperitoneal administration of edaravone after HIE for consecutive days improved memory and learning ability when given in the acute phase of HIE [111].

### ***Current Clinical Trial Studies***

## **Erythropoietin**

Erythropoietin (Epo) is a 34 kilodalton glycoprotein with pleiotropic properties. Epo has been reported to have effects on a variety of receptor-mediated and cell-specific mechanisms that are beneficial and essential after HIE. Epo has been associated with anti-inflammatory, anti-excitotoxic, anti-oxidative, and anti-apoptotic effects as well as promoting neurogenesis and angiogenesis [72,73,74]. Epo is expressed in both human and animal brains in its early development but decreases gradually after birth [112].

Hypoxia-inducible factor-1 mediates the increase of Epo after HIE in the brain, leading not only to an increase in Epo expression but also an increase in the Epo receptor in neurons, astrocytes, and microglia [75,76]. Epo levels are increased in newborn infants with HIE in the cerebrospinal fluid despite the absence of exogenous Epo treatment

[112]. In the setting of HIE there is an increase in permeability of the blood brain barrier [113,114,115], allowing high doses of Epo to increase its levels in the CSF [113,114,115,116]. Studies have shown that neonatal rats with HIE injuries have histological and functional improvements following high-doses of Epo and that multiple doses reduces infarct volumes in a dose-dependent manner [118]. Kumral et al. demonstrated that a single dose of Epo (1000 U/kg i.p.) immediately after neonatal hypoxic–ischemic insult diminished long-term spatial memory deficits. In addition, a treatment group that received Epo but did not undergo HIE, showed no differential effects concerning learning or memory from the treatment [119]. One clinical study also showed that Epo 1000 and 2500 U/kg per dose intravenously administered along with hypothermia achieved and surpassed plasma concentrations that provided neuroprotection in animal models [118]. Furthermore a study evaluating middle cerebral artery occlusion in neonatal rats found that three daily doses of Epo (1 U/g, i.p. each) caused increases in hemispheric volume and its sub regions, as well as spatial learning and memory [120].

There have been several completed clinical trials concerning Epo after HIE injury [114,121,122,123]. Currently there are two active clinical trials (NCT01913340 and NCT01732146) examining Epo in combination with hypothermia in infants with HIE. The “Neonatal Erythropoietin And Therapeutic Hypothermia Outcomes in Newborn Brain Injury” study (NCT01913340) assesses an Epo dose of 1000 U/kg/dose IV  $\times$  5 doses. While the “Efficacy of Erythropoietin to Improve Survival and Neurological Outcome in Hypoxic Ischemic Encephalopathy” study (NCT01732146) evaluates Epo intravenous injections (5000 U/0.3 mL) 1000 to 1500 U/kg/dose three times given every 24 h with the first dose within 12 h of delivery.

Epo exerts neuroprotection through phosphorylation of its receptor and Janus Kinase 2, which provides a docking complex for intracellular signaling proteins including PI3K as well as Akt, signal transducer and activator of transcription 5 (STAT5), and the extracellular signal-regulated kinase (ERK). Activating these pathways leads to alteration of cell proliferation, survival, and differentiation by affecting a number of downstream targets. For example, Akt limits inflammation [124] and decreases apoptotic cell death. STAT5 acts on cell survival [125], while the ERK pathway has demonstrated not only to have anti-apoptotic and anti-inflammatory effects in vitro but also to be essential for neurogenesis and cell fate commitment [126,127].

### **Allopurinol**

Allopurinol is a xanthine oxidase inhibitor that lowers uric acid concentration in patients with gout and neoplastic diseases. In addition, allopurinol functions as a chelator of non-protein bound iron as well as a direct scavenger of hydroxyl radicals, suggesting it may serve in neuroprotection [128]. Indeed, allopurinol provided neuroprotection in postnatal day 7 rats after HIE [77]. Even though Benders and colleagues did not show improvement in outcomes with after-birth asphyxia [129], a recent follow-up study in human neonates with allopurinol used on term asphyxiated neonates showed benefits on mortality and severe disabilities at 4–8 years of age [130]. In addition, Kaandorp and colleagues investigated the pharmacological applicability of allopurinol for intrauterine neuroprotection after maternal administration. They showed intravenously administered allopurinol to the mother rapidly crosses the placenta with satisfactory concentrations reaching the neonate at birth. In addition, it is safe to both the mother and the neonate.

In 2014, the same group published the results of another clinical trial. Maternal treatment with allopurinol during fetal hypoxia did not significantly lower neuronal damage markers in umbilical cord blood. However, post hoc analysis revealed a potential benefit in treatment of females (NCT00189007) [78]. There is an ongoing study investigating the reduction in free radical formation after reperfusion with initiation of this medication during labor, with the intention of reducing free radical induced post asphyxia brain damage. They hope to demonstrate how allopurinol during asphyxia reduces post-hypoxic-ischemic reperfusion damage in the newborn (NCT00189007). In addition, there is another ongoing trial (the European ALBINO trial) that will assess outcomes at 2 years of life. Studies have shown allopurinol is a viable treatment option for early fetal neuroprotective therapy during labor, but future studies and clinical investigation are necessary to further support its effectiveness.

## **Xenon**

Xenon is a potent anesthetic with a low gas partition coefficient. It crosses the BBB easily and guarantees rapid induction of anesthesia. As an anesthetic it has proven to be safe in adults and well tolerated [131]. However, a recent review by Istaphanous and Loepke demonstrated that xenon may trigger neurodegeneration in the developing brain [132]. Thus, the safety of a newborn injured brain is not expected.

Important neuroprotective effects of Xenon have been demonstrated in adult rats in transient brain ischemia [79]. In neonatal rodents, it was associated with relatively limited protection when given alone but did protect for up to 30 days when given in

combination with hypothermia [80]. Similarly, other studies have also proven this combination to be beneficial [133,134].

Xenon was reported to be safe for use in a phase II randomized study outcomes after demonstrating to have similar results as cooling therapy alone [135]. An ongoing clinical trial (NCT01545271), estimated to be completed in October 2015, aims to examine the effect of inhaled xenon gas in the treatment of newborn infants with HIE in combination with cooling, which is the standard treatment of this condition. They hypothesize that the xenon and cooling combination will produce better neuroprotection than the standard treatment of cooling alone. Hypothermia plus adjuvant therapies have been extensively reviewed in two recent publications [136,137]. Based on the preclinical studies, ongoing trials in neonates include inhaled Xenon and cooling (NCT01545271 and NCT00934700).

### **Topiramate**

Topiramate (TPM) is an anticonvulsant agent with multiple mechanisms of action [138,139], implying its ability to be a neuroprotective agent. It has neuroprotective qualities according to previous literature. Its neuroprotective mechanisms appear to be related not only to AMPA and Kainate receptors inhibition [81,82,83,140,141] but also to blockade of Na<sup>+</sup> channels [84], high voltage-activated calcium currents [85], carbonic anhydrase isoenzymes [86], and mitochondrial permeability transition pore [87].

Even though no clinical studies have been published to prove an additive or synergistic action of TPM in concert with hypothermia in newborns, ongoing clinical trials (NCT01765218), Topiramate in Neonates Receiving Whole Body Cooling for

Hypoxic Ischemic Encephalopathy, are investigating whether topiramate improves the outcomes of babies with neonatal hypoxic encephalopathy who are receiving whole body cooling. This trial is to be completed in 2017.

TPM in concert with melatonin decreases infarcted volume and apoptosis in neonatal HIE rat model [88]. In addition, Noh and colleagues [89] reported that i.p. or per oral topiramate pretreatment significantly reduced the brain damage and subsequent cognitive impairments induced by hypoxia-ischemia in neonatal rats. Similarly, it leads to dose dependent and long lasting neuroprotection in the excitotoxic newborn mouse model [141]. Topiramate is able to provide neuroprotection by increasing survival of pre-oligodendrocytes, decreasing neuronal apoptosis, inhibiting microglial activation and astrogliosis, and decreasing seizure activity.

Melatonin and topiramate, acting on different stages of HIE, used alone or in combination, significantly decreased the percent infarcted area, and apoptotic cell death in neonatal HIE rat model. It is necessary to investigate different doses and application times of these agents as combination therapy in order to provide more effective neuroprotection. Furthermore, an ongoing trial: The NeoNATI trial (NCT01241019) will evaluate neurological outcomes at 6, 12, and 18 months of life and help clarify questions as to whether the administration of TPM in newborns with HIE potentiates the neuroprotective effect of treatment with hypothermia. They hypothesize that the combination treatment with moderate whole-body hypothermia associated with TPM administration is safe and enhances the neuroprotective properties of hypothermia for the treatment of neonatal HIE.

## **Magnesium Sulfate**

Magnesium Sulfate (MgSO<sub>4</sub>) has gained a lot of interest in the research community due to its ability to alleviate excitotoxic damage in vitro by binding to the magnesium site on N-methyl-d-aspartate (NMDA) glutamate channel [142]. Evidence leads researchers to believe that it also reduces secondary inflammation and associated injury [143], acts on cell membrane stabilization and inhibition of free radical production [144], and improves cardiovascular stability [145].

MgSO<sub>4</sub> is also known to be neuroprotective. However, controversy regarding its efficacy in protecting the brain in term infants who may suffer encephalopathy exists. These thoughts emerged due to the fact that the outcomes of previous studies are highly inconsistent when it comes to neuroprotection. Differences in dose and timing of administration were present amidst evidence of beneficial effects [90].

Robert Galinsky and colleagues showed that the effect of MgSO<sub>4</sub> treatment before or shortly after acute HIE at term or near-term equivalent was highly inconsistent between studies [90]. This caused questions and concerns to arise regarding the benefits of MgSO<sub>4</sub> since the perinatal studies on this topic did not directly control brain or body temperature, yet suggested beneficial effects of MgSO<sub>4</sub>. In addition, most of these rodent studies didn't control environmental temperatures. The studies in which the body temperature was controlled in large animal translational models suggested lack of effect after 2 or 3 days of recovery [146,147,148].

Tagin and colleagues also demonstrated that there is insufficient evidence to determine if magnesium therapy given shortly after birth to newborns with HIE reduces death or moderate-to-severe disability [91]. Currently, an ongoing phase III clinical trial

(NCT01646619) is assessing whether the addition of a drug such as MgSO<sub>4</sub> while providing therapeutic hypothermia or cooling to babies who are asphyxiated at birth provides additional benefit to the survival and outcomes compared to cooling alone. Severe Neurodevelopmental Disability will be assessed at discharge from the hospital and at 18–24 months of age to assess developmental delay and cerebral palsy.

There is insufficient evidence to determine if magnesium therapy given shortly after birth to newborns with HIE reduces death or moderate to severe disability. The improvement in short-term outcomes without significant increase in adverse effects supports the need for further adequately powered trials to determine if there are long-term benefits of magnesium and to confirm its safety. Mortality should be monitored closely in all future trials involving magnesium therapy for newborns with HIE. In the current review, the results, although statistically insignificant for mortality between the magnesium and the control groups, showed a trend toward an increase in mortality in the magnesium group.

### **Stem Cell Therapy and Neonatal HIE**

Stem cell therapy represents a modern cornerstone of promising neuroprotective and neuroregenerative treatment options that can benefit from ongoing trials, especially in adult stroke [92]. However, in context of perinatal HIE, it has gained importance as adjunct treatment with hypothermia in recent clinical trials to meliorate mortality and chronic neurological disability. Several sources for stem cells include neural stem/progenitor cells derived from fetal tissue, mesenchymal stem cells or embryonic stem-induced pluripotent stem cells [93,94] (Figure 3).



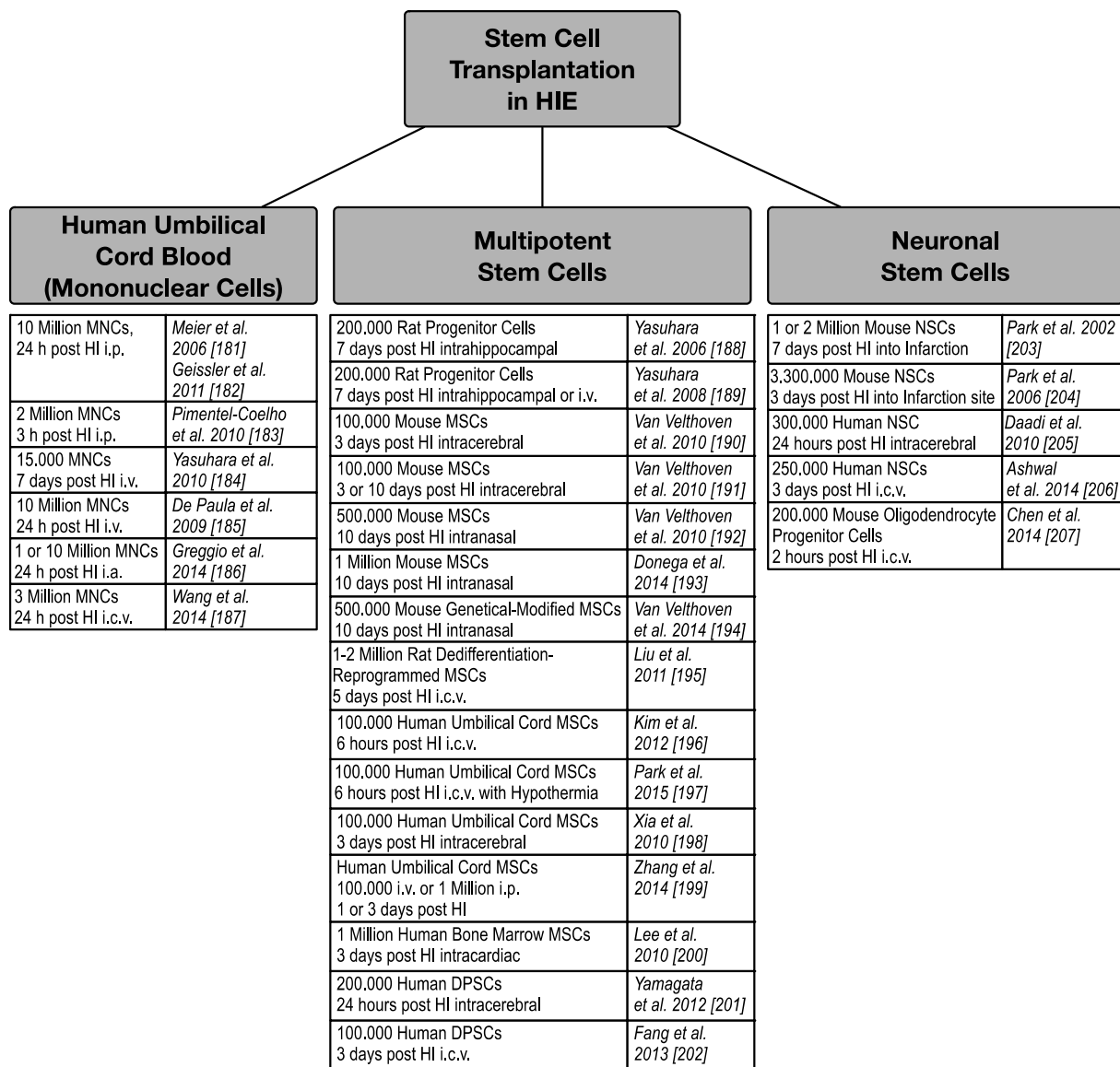


Figure 3. Stem Cell Transplantation in Animal Models of HIE. Summary of stem cell transplantation studies in various animal models of HIE. Human Dental Pulp Stem Cells (DPSCs); Hypoxic Ischemic Encephalopathy (HIE); Mononuclear Cells (MNCs); Mesenchymal Stem Cells (MSCs); Neuronal Stem Cells (NSC); Based upon cell dose, cell type, transplantation timing, and administration route.

Additionally, cord blood (CB) represents a rich source of stem cells used in several animal models of neurological diseases [93,94,95,96,149]. Autologous transplantation of CB, collected shortly after delivery, has the advantages of minimal ex vivo manipulation, no necessary immunosuppression, relatively easy access, and storage properties. CB is rich in primitive stem cells, yet it contains a limited number of cell types, mostly mononuclear cells, and showed to be not as pluripotent as embryonic stem cells [97]. Studies analyzing the risk and benefits of autologous CB infusion in neonates with HIE and in children with cerebral palsy show promising results [97,150,151]. Placebo-controlled clinical trials are demanded. Currently ongoing clinical trials include the initiated trial by Cotten et al. of autologous CB infusion in term infants with HIE (NCT00593242) [97,152].

Due to the lack of imaging diagnostic difficulties to detect HIE in premature newborns and insufficient data, present stem cell therapy trials are restricted to full term infants [153,154]. Further investigation is needed for developing the best strategy considering transplantation timing, cell dosage, ex vivo modulations, way of administration, and choice of stem cells [97]. It should be mentioned that, besides stem cell transplantation, there is research ongoing in the field of stem cell factors. G-CSF [3], and glial-cell derived neurotrophic factor have shown promising results [155].

As reviewed above, the complex etiology of HIE requires treatment that will act on multiple processes [156]. There is an important unmet need to further improve the outcome of neonatal encephalopathy in term infants. The agents mentioned in this section either alone or in combination deserve rigorous and focused testing in order to render better results that would allow researchers to translate the studies to clinical scenarios.

Optimal dose, route, and duration of administration are still parameters that need to be researched in depth in order to provide better guidance about the next step to follow. Any favorable results might lead to new perspectives leading to reduction of cerebral damage in asphyxiated newborns. Intensive tests are needed to provide a platform for furthering clinical trials to better support their use in the clinical setting and answering many questions that remain to be answered (Figure 4).

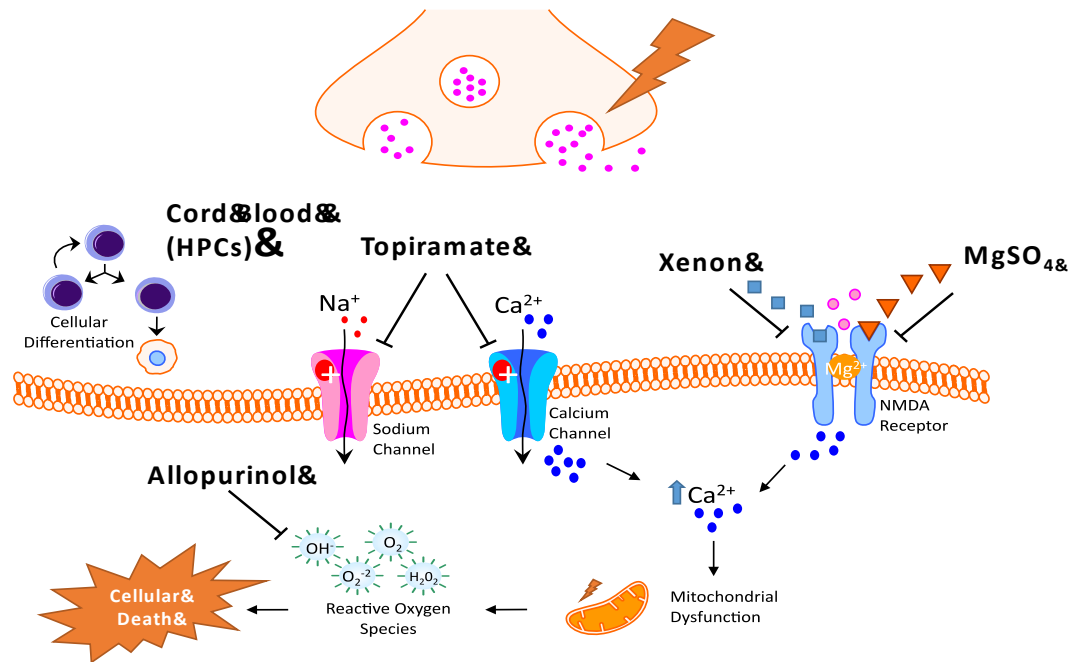


Figure 4. Proposed Mechanisms of Current Clinical Trials. Cord blood infusions are rich with hematopoietic stem cells and neurotrophic factors that have numerous effects such as immunomodulation, reduction of microglia and T-lymphocyte infiltration, as well as the potential to increase neurogenesis and angiogenesis. It is believed that topiramate is able to block sodium channels and high voltage-activated calcium currents after HIE. Xenon is believed to bind at the glycine site of the NMDA receptor and inhibit its downstream effects. Similarly magnesium sulfate also inhibits the NMDA receptor by binding to the magnesium site of the receptor. Allopurinol is predicted to provide neuroprotection by directly scavenging hydroxyl radicals after HIE injury.

### **Central Hypothesis**

Thus our central hypothesis is to evaluate the efficacy of neuroprotective molecules in reducing apoptosis and inflammation after neonatal HIE. As well as characterizing the mechanistic actions of neuroprotection of these molecules and how they interact and effect HIE pathophysiology.

### **Significance**

The significance of these projects will be to provide a basis for clinical translation of these neuroprotective molecules. This project also has the possibility to make strides in the intricate task of improving the rate of mortality and the global quality of care since neonatal hypoxic ischemic encephalopathy has a tremendous effect on the surviving neonates, their families, and society as a whole.

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**CHAPTER TWO**

**INTRANASAL ADMINISTRATION OF INTERFERON BETA ATTENUATES  
NEURONAL APOPTOSIS VIA THE JAK1/STAT3/BCL-2 PATHWAY IN A RAT  
MODEL OF NEONATAL HYPOXIC-ISCHEMIC ENCEPHALOPATHY**

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The work presented in this chapter has been published.  
ASN Neuro. 2016 Sep 28;8(5).

## Abstract

Neonatal hypoxic-ischemic encephalopathy (HIE) is an injury that often leads to detrimental neurological deficits. Currently, there are no established therapies for HIE and it is critical to develop treatments that provide protection after HIE. The objective of this study was to investigate the ability of interferon beta (IFN $\beta$ ) to provide neuroprotection and reduce apoptosis after HIE. Postnatal Day 10 rat pups were subjected to unilateral carotid artery ligation followed by 2.5 hr of exposure to hypoxia (8% O<sub>2</sub>). Intranasal administration of human recombinant IFN $\beta$  occurred 2 hr after HIE and infarct volume, body weight, neurobehavioral tests, histology, immunohistochemistry, brain water content, blood–brain barrier permeability, enzyme-linked immunosorbent assay, and Western blot were all used to evaluate various parameters. The results showed that both IFN $\beta$  and the Type 1 interferon receptor expression decreases after HIE. Intranasal administration of human recombinant IFN $\beta$  was able to be detected in the central nervous system and was able to reduce brain infarction volumes and improve neurological behavior tests 24 hr after HIE. Western blot analysis also revealed that human recombinant IFN $\beta$  treatment stimulated Stat3 and Bcl-2 expression leading to a decrease in cleaved caspase-3 expression after HIE. Positive Fluoro-Jade C staining also demonstrated that IFN $\beta$  treatment was able to decrease neuronal apoptosis. Furthermore, the beneficial effects of IFN $\beta$  treatment were reversed when a Stat3 inhibitor was applied. Also an intraperitoneal administration of human recombinant IFN $\beta$  into the systemic compartment was unable to confer the same protective effects as intranasal IFN $\beta$  treatment.



## **Introduction**

Neonatal hypoxic-ischemic encephalopathy (HIE) is a devastating disease that primarily causes neuronal and white matter injury. HIE has tremendous detrimental effects on the developing brain and is among the leading causes of death among infants, as well as the major underlying cause of seizures in term infants (Volpe, 2001; Doycheva et al., 2013; Shetty, 2015). Although there have been major advances in modern technology and an increased understanding of fetal and neonatal pathologies, HIE is still a serious condition that is unresolved and causes significant mortality and longterm morbidity (Badr Zahr and Purdy, 2006; Gill and Perez-Polo, 2008; Northington et al., 2011; Shankaran, 2012). These adverse events in the developing brain often lead to long lasting detrimental neurological defects later on in life such as mental retardation, epilepsy, cerebral palsy, learning disabilities, and other neurophysiological handicaps (Fathali et al., 2010; Li et al., 2016). Currently, there are no specific treatments to repair the damage caused by HIE (Zhu et al., 2013; Shaikh et al., 2015). Thus, it is critically important to develop safe and effective therapies (Ramanantsoa et al., 2013; Caltagirone et al., 2016).

Neonatal HIE can also be characterized as an injury that occurs in the immature brain, resulting in delayed cell death via excitotoxicity, inflammation, and oxidative stress (Bain et al., 2013; Dixon et al., 2015). Previous studies have shown that the newborn brain is primed to respond to various insults with the activation of apoptotic cascades since cell death is a normal part of development in the central nervous system (CNS). Thus, there is a high expression of pro-apoptotic proteins in the developing brain (Northington et al., 2011). As a consequence, mitochondrial dysfunction occurs and

ultimately signals pathways of apoptosis (Qi et al., 2015; Alhadidi et al., 2016). Specifically, the release of cytochrome c by the mitochondria leads to activation of caspase-9 followed by active caspase-3 between 6 and 48 hr after injury (Gill and Perez-Polo, 2008). Clinically, interferon beta (IFN $\beta$ ) is the primary treatment used to combat inflammation and flare-ups in multiple sclerosis (Johnston and So, 2012; Castrop et al., 2013). IFN $\beta$  is able to increase expression and concentration of anti-inflammatory cytokines, while also having the effect of decreasing the expression of pro-inflammatory agents (Kieseier, 2011). However, the neuroprotective properties and mechanisms of IFN $\beta$  have not yet been explored following HIE. The administration of human recombinant IFN $\beta$  as a treatment is a novel approach in neonatal HIE since it is already a food and drug administration approved treatment for multiple sclerosis (English and Alois, 2015). Intrastriatal injections of IFN $\beta$  have been shown to preserve the blood–brain barrier integrity, decrease infarct size, and block the infiltration of inflammatory cells in an adult rat model of middle cerebral artery occlusion (Veldhuis et al., 2003). These studies indicate that IFN $\beta$  may have some anti-apoptotic and anti-inflammatory properties in the CNS after significant damage like HIE. IFN $\beta$  specifically acts through the Type 1 interferon receptor (IFNR) which has been shown to be expressed on endothelial cells and leukocytes (Ross et al., 2004; Johnston and So, 2012). It has been characterized that after IFNR activation, the Jak-Stat pathway is triggered for positive feedback of IFN $\beta$  and activation of several other pathways that are associated with anti-apoptosis (Heim, 1999; Schindler et al., 2007; Zula et al., 2011). As a result of IFN $\beta$  binding to the receptor, one product in neurons is Stat3 (Schindler et al., 2007; Dziennis and Alkayed, 2008; Ramgolam et al., 2011). It has been shown that Stat3 leads to the

increased transcription of Bcl-2, which lowers the Bax/Bcl-2 ratio (Dziennis and Alkayed, 2008). Interestingly, p53 increases after HIE injury and induces the increase of Bax (Northington et al., 2011). This causes a high Bax/Bcl-2 ratio leading to Bax-mediated mitochondrial permeabilization, which is also known to increase after HIE (Wullner et al., 1998; Pan et al., 2012; Thornton and Hagberg, 2015). These events can lead to the activation and cleavage of caspases, specifically caspase-9 and caspase-3 (Wullner et al., 1998; Pan et al., 2012; Thornton and Hagberg, 2015). Thus, we were able to derive the hypothesis that intranasal delivery of human recombinant IFN $\beta$  will provide protection through its anti-apoptotic properties after HIE.

## **Materials and Methods**

All protocols and experiments in this study were approved by the Institutional Animal Care and Use Committee of Loma Linda University. All animals were handled in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals.

### ***Neonatal Hypoxic Ischemic Encephalopathy Animal Model***

A modified Rice-Vannucci model was used as previously described in our past publications (Fathali et al., 2013; Drunalini Perera et al., 2014; Charles et al., 2015). Postnatal Day 10 Sprague-Dawley rat pups purchased from Harlan Laboratories (Livermore, CA) were anesthetized and underwent a unilateral right common carotid artery ligation using a 5-0 surgical silk suture. After a recovery period of 1 hr, the rat pups were placed in a submerged 37 hypoxia chamber (8% O<sub>2</sub>) for 2.5 hr. The flow rate

of the gas 93.82 mL/min for the first 1.25 hr and was 77.30 mL/min for the remaining 1.25 hr. Sham animals also underwent anesthesia and exposure of the carotid artery; however, the artery was not ligated.

### ***Postsurgical Care***

After the surgeries, the rat pups were returned to their mothers. The rat pups were monitored closely for bleeding, swelling, pain, and distress. The monitoring occurred every 20 min for the first 2 hr, at 6 hr, and then once daily until sacrifice.

### ***Drug Administration***

Intranasal administration of IFN $\beta$  (0.03 mg/kg, 0.3 mg/kg, and 1.0 mg/kg) was applied 2 hr after HIE in the treatment group. The best dose (0.3 mg/kg) was chosen and administered every 24 hr until the 72-hr time point and then was 2 ASN Neuro used throughout the duration of the study. A specific Stat3 inhibitor, WP1066 (0.5 mg/kg), was applied via intraperitoneal injection 1 hr before HIE in combination with IFN $\beta$  treatment. In addition, one group of animals received 0.3 mg/kg IFN $\beta$  treatment via intraperitoneal injection. The vehicle used to dissolve the IFN $\beta$  tablets was deionized water which was administered to the sham and vehicle groups. Human recombinant IFN $\beta$  (Avonex) was purchased from the Loma Linda University Medical Center Pharmacy (Loma Linda, CA), and WP1066 was obtained from Abcam (Cambridge, MA).

### ***Intranasal Administration***

Intranasal administration was performed as previously described (Topkoku et al.,

2013; Zhang et al., 2015). Rat pups were anesthetized with isoflurane and placed on their backs in an anesthesia chamber. Underneath the anesthesia chamber, a heating pad was used to maintain the temperature. A rolled pad was placed under the necks of the rat pups to keep the heads stable. After the treatment, the rat pups were monitored until they were fully conscious and exhibited normal breathing.

### ***2, 3, 5-Triphenyltetrazolium Chloride Monohydrate Staining***

At the 24- and 72-hr time points, the rat pups were anesthetized and perfused transcardially with phosphate-buffered saline (PBS). The brains were removed, sectioned into 2 mm slices, and immersed into a 2% 2, 3, 5-Triphenyltetrazolium Chloride Monohydrate (TTC) solution at 37C for 5 min as routinely performed (Chen, Burris, et al., 2011; Dock et al., 2015). The samples were then rinsed with PBS and stored in a 10% formaldehyde solution or underwent preparation for Western blot to be utilized in the protein expression evaluation groups as previously described (Kramer et al., 2010). The infarct volume was traced and analyzed with Image J Software.

### ***Western Blot***

The Western blot experiments were executed as routinely performed (Zheng et al., 2015; Sherchan et al., 2016). Rat pups were euthanized under isoflurane followed by the preparation of brain tissue samples. Tissues samples were created by collecting and snap freezing the brain hemispheres with liquid nitrogen followed by storage at 80C. Prior to the Western blot sample preparation, these samples were stained with TTC since they are shared with the infarct evaluation groups. Cytosolic fractionation extracts were

obtained from the brain samples. Denatured protein extract (50 mg) was electrophoresed and transferred to a nitrocellulose membrane and probed with antibodies. The following primary antibodies were used: Interferon beta-receptor (1:500; Santa Cruz Biotechnology), IFN $\beta$  (1:500; Santa Cruz Biotechnology), Stat-3 (1:500; Abcam), Bcl-2 (1:1000; Cell Signaling), and cleaved caspase 3 (1:500; Cell Signaling). The membranes were then incubated with the appropriate secondary antibodies (Santa Cruz Biotechnology and Thermo Fisher). The optical densities of the bands were visualized using ECL Plus (GE Healthcare Life Sciences) or Li-Cor fluorescence technology and were analyzed with Image J Software (National Institute of Health).

#### ***Enzyme-Linked Immunosorbent Assay (ELISA) Assay***

Rat pups were euthanized under isoflurane anesthesia, and the brain hemispheres were collected, snap frozen in liquid nitrogen, and stored at 80C. Human recombinant IFN $\beta$  from the cytosolic fractionation extracts was then detected using a commercial ELISA kit (Thermo Scientific).

#### ***Histology and Immunohistochemistry***

Immunohistochemistry was conducted as routinely executed (Chen, Zhang, et al., 2015; Huang et al., 2015). The rat pups were perfused under anesthesia with PBS followed by 4% formaldehyde. The brains were removed for postfixation in formalin. The paraffin-embedded brains were then sectioned into 10  $\mu$ m slices via cryostat. The brain slices were then evaluated with immunohistochemistry and a Fluoro-Jade C staining kit (Biosensis). The specific cellular marker antibody used was NeuN (1:100;

Abcam). The brain slices were then incubated with the appropriate fluorescent secondary antibodies (Jackson ImmunoResearch). The brain slices were then visualized with a fluorescent microscope (Olympus BX51 and Keyence BZ-9000) under 20x, 40x, and 60x magnifications with an aperture of 0.75, 0.95, and 0.95, respectively.

### ***Short-term Neurobehavioral Tests***

Both the righting reflex test and the geotactic reflex test were performed as previously described (Zhang et al., 2013; Yuan et al., 2014). Both short-term neurobehavioral tests were conducted at the 24-hour time point and continued to the 72-hour time point after HIE.

**Righting Reflex Test:** The righting reflex test was performed by placing the pup in a supine position on a table surface and recorded the amount of time for the animal to reach a prone position, where all four paws are against the surface of the table.

**Geotactic Reflex Test:** The geotactic reflex test was performed by placing the rat pups in a downward orientation on a 45-degree incline. The amount of time for the pup to recognize its position on an incline, make a full 180-degree turn, and face the top of the incline was recorded.

### ***Brain Water Content***

Brain water content was performed as previously conducted (Chen et al., 2009; Li, Tao, et al., 2015). The rat pups were placed under isoflurane anesthesia and were decapitated 24 hr after injury, and the brain hemispheres were quickly separated and removed. All tissue samples were weighed using an analytical microbalance in order to

obtain the wet weight. The samples were then dried at 100°C for 24 hr before determining the dry weight. Brain water content (%) was calculated as (wet weight - dry weight)/dry weight x 100.

### ***Evan's Blue Dye Extravasation Assay***

Evan's Blue dye extravasation was executed as previously described (Li, McBride, et al., 2015; Merali et al., 2015; Dixon et al., 2016; Zhang et al., 2016). Evan's Blue dye was administered via intraperitoneal injection and allowed to circulate 6 hr before the 24-hr time point to measure blood–brain barrier permeability. At the 24-hr time point, the rat pups were sacrificed under anesthesia and perfused with PBS. The brain hemispheres were then collected and homogenized in a 3:1 ratio of 50% trichloroacetic acid to brain tissue mass. The supernatant was then analyzed at 620 nm by spectrophotometry.

### ***Statistical Analysis***

T-test comparisons, one-way analysis of variance, Newman-Keuls multiple comparison test, and Tukey's post hoc analysis were performed in GraphPad Prism.

## **Results**

### ***Expression of IFN $\beta$ and IFNR in the CNS After HIE***

IFNR expression decreases shortly after HIE insult and is also significantly different from the naïve control group after 24 hr and 72 hr (Figure 1(a)). IFN $\beta$  significantly decreases after HIE at the 72-hr time point when compared with the naïve



control group (Figure 1(b)). There are no significant differences in protein expression 0 to 72 hr after HIE for both IFN $\beta$  and IFNR (IFN $\beta$ : N = 3 rats per group; IFNR: N = 4 rats per group). Significance determined based on comparison to the naïve control group (\*p < .05) as calculated by student's T test.

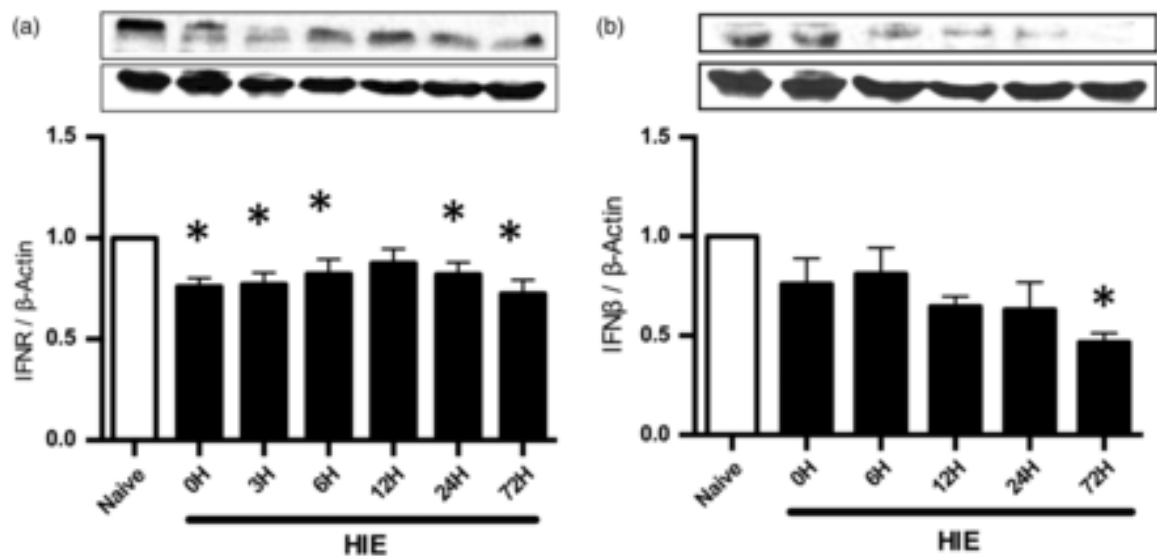


Figure 5: Endogenous Type 1 interferon receptor and interferon beta expression in the injured hemisphere of the neonatal central nervous system. (a) Western blot analysis of the injured brain hemisphere illustrates that IFNR expression decreased after 24 hr in the central nervous system. (b) Western blot analysis of the injured brain hemisphere demonstrates that IFNβ protein expression decreases at 72 hr after injury. Significance determined based on comparison to the naïve control group (\* $p < .05$ ) as calculated by student's T test. HIE = hypoxic-ischemic encephalopathy; IFNβ = interferon beta.

### ***Exogenous IFN $\beta$ Reaches the CNS After Intranasal Administration***

A time course of a single dose of human recombinant IFN $\beta$  intranasal administration was performed. Intranasal IFN $\beta$  0.3  $\mu$ g/kg (N = 6 rats per group) significantly increased 1 hr after the initial administration, and peak concentrations were observed at the 12-hr time point. IFN $\beta$  began to decline after 24 hr; however, it was still significantly higher than the control group (Figure 2(a)). Significance determined based on comparisons to the naïve control group and the IN-1H group (\*p < .05 vs. Naïve; #p < .05 vs. IN-1H) as calculated by the Newman-Keuls multiple comparison test.

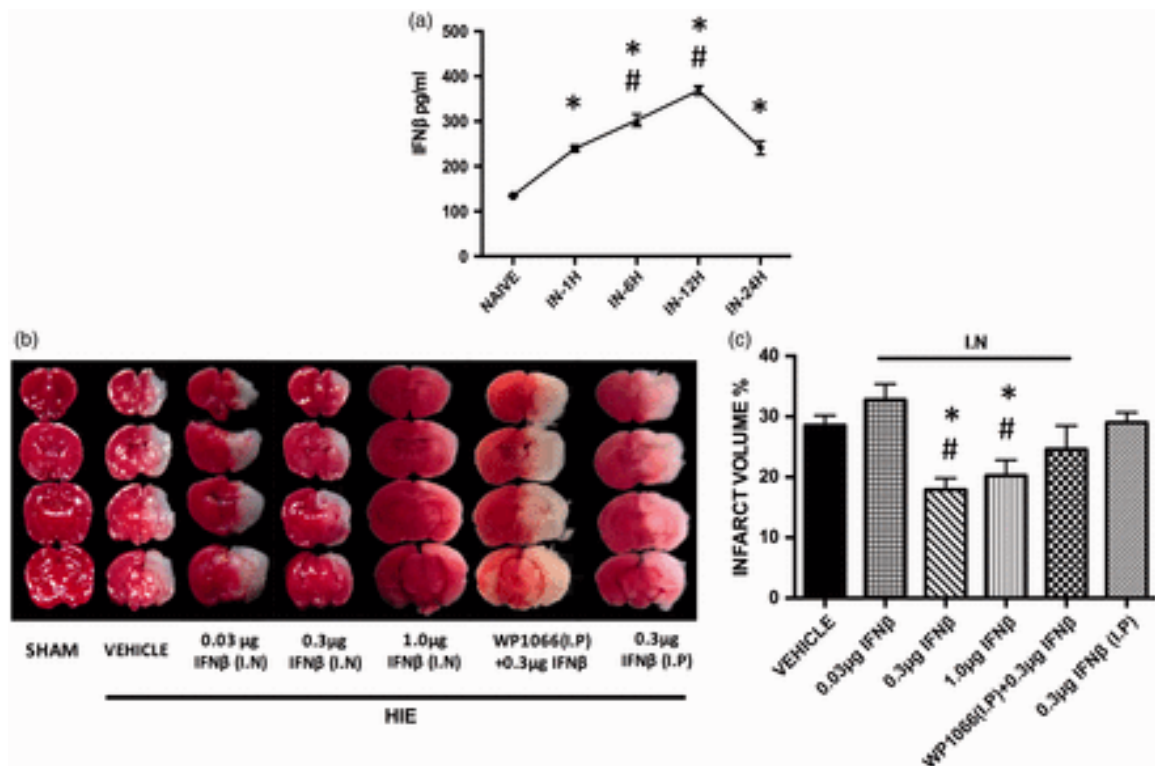


Figure 6: Intranasal administration of human recombinant interferon beta after 24 hr in the central nervous system. (a) A time course of exogenous IFN $\beta$  in the injured right hemisphere of the central nervous system after intranasal administration using an ELISA assay. Significance determined based on comparisons to the naïve control group and the IN-1H group (\* $p < .05$  vs. Naive; # $p < .05$  vs. IN-1H) as calculated by the Newman-Keuls multiple comparison test.

(b) Illustrates the effects of IFN $\beta$  intranasal treatment with TTC staining of 2 mm brain slices 24 hr after neonatal hypoxic-ischemic encephalopathy. (c) Displays that intranasal administration 0.3  $\mu$ g and 1.0  $\mu$ g IFN $\beta$  decreases infarct size 24 hr after injury. Intraperitoneal administration of IFN $\beta$  did not reduce brain infarct volume. The effect of intranasal IFN $\beta$  was reversed when coadministered with a Stat3 inhibitor (WP1066). Significance determined based on comparisons to the HIE+Vehicle and HIE+0.03  $\mu$ g IFN $\beta$  groups (\* $p < .05$  vs. HIE+Vehicle; # $p < .05$  vs. HIE+0.03  $\mu$ g IFN $\beta$ ) as calculated by Tukey's test. HIE = hypoxic-ischemic encephalopathy; IFN $\beta$  = interferon beta.

### ***Intranasal Administration of IFN $\beta$ Decreased Infarct Volume 24 hr after HIE***

Intranasal IFN $\beta$  medium, HIE + 0.3  $\mu$ g IFN $\beta$  (N = 10 rats per group) and high dose HIE + 1.0  $\mu$ g IFN $\beta$  (N = 6 rats per group) treatments were administered at 2 hr following HIE. Both doses were able to decrease infarct volumes after 24 hr when compared with the other control groups (\*p < .05 vs. HIE + Vehicle; #p < .05 vs. HIE + 0.03  $\mu$ g IFN $\beta$ ; Figure 2(b) and (c)). The low dose group, HIE + 0.03  $\mu$ g IFN $\beta$  (N = 6 rats per group), had no effect on infarct volume when compared with the HIE + Vehicle group after 24 hr. Significance determined based on comparisons to the HIE + Vehicle and HIE + 0.03  $\mu$ g IFN $\beta$  groups (\*p < .05 vs. HIE + Vehicle; #p < .05 vs. HIE + 0.03  $\mu$ g IFN $\beta$ ) as calculated by Tukey's test.

### ***Intraperitoneal Administration of IFN $\beta$ as well as Cotreatment of a Stat3 Inhibitor and***

#### ***Intranasal IFN $\beta$ Fails to Reduce Infarct Volumes 24 hr After HIE***

Intraperitoneal injection of IFN $\beta$  (0.3  $\mu$ g) was ineffective in reducing infarct volume 24 hr after injury (N = 4 rats per group). Also the protective effects of intranasal administration of IFN $\beta$  were diminished when WP1066 (0.5  $\mu$ g/kg), a Stat3 inhibitor, was administered in combination with IFN $\beta$  treatment at 24 hr post injury. The HIE + WP1066 + 0.3  $\mu$ g IFN $\beta$  group (N = 5 rats per group) was not significantly different from the HIE + Vehicle group (Figure 2(b) and (c)).

### ***Intranasal Administration of IFN $\beta$ Has no Effect on Brain Water Content and Blood–***

#### ***Brain Permeability at 24 hr after HIE***

Figure 3(a) shows the trend that IFN $\beta$  treatment can reduce brain water content at

the 24-hr time point (N = 7 per group). Brain water content in the injured right hemisphere was significantly increased in both the HIE + Vehicle and HIE + IFN $\beta$  groups when compared with sham controls. Significance determined based on comparisons to the Sham group (\*p < .05 vs. Sham) as calculated by Tukey's test. This indicates that at 24 hr, IFN $\beta$  treatment may not play a strong role in reducing brain edema but plays a more anti-apoptotic role. Similarly, Figure 3(b) also shows the trend that IFN $\beta$  treatment may decrease blood–brain barrier permeability (N = 7 per group). No significance was determined as calculated by Tukey's test.

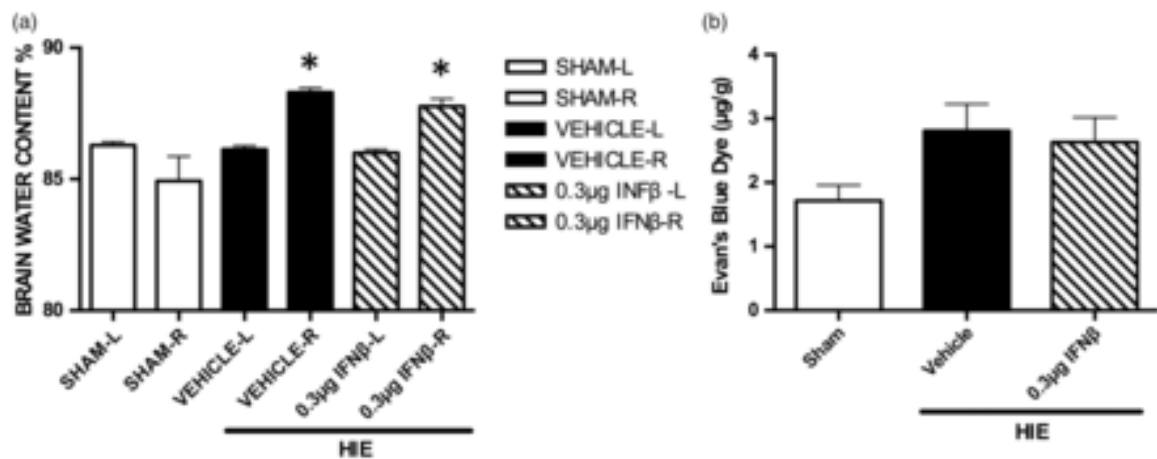


Figure 7: The effects of intranasal administration of human recombinant interferon beta on brain water content and blood barrier permeability after 24 hr after hypoxic-ischemic encephalopathy. (a) Displays the brain water content of each hemisphere of the brain 24 hr after hypoxic-ischemic encephalopathy, indicating brain edema in the injured right hemisphere. Significance determined based on comparisons to the Sham group (\* $p < .05$  vs. Sham) as calculated by Tukey's test. (b) The amount of Evan's Blue extravasation in the hemispheres of the central nervous system after 24 hr after hypoxic-ischemic encephalopathy. No significance was determined as calculated by Tukey's test. HIE = hypoxic-ischemic encephalopathy; IFNβ = interferon beta.

***Intranasal Administration of IFN $\beta$  Decreases Infarct Volume and Improves Short-Term Neurobehavioral 72 hr after HIE***

Daily intranasal administration of IFN $\beta$  0.3  $\mu$ g treatment (N = 7 rats per group) also significantly reduced infarct volume when compared with the HIE + Vehicle group (N = 7 rats per group) at the 72-hr time point as well (Figure 4(a) and (b)). Significance determined based on comparisons to the HIE + Vehicle group (\*p < .05 vs. HIE + Vehicle) as calculated by student's T test. The daily administration of 0.3  $\mu$ g IFN $\beta$  treatment was able to improve weight loss after injury (Figure 4(c) and (d)). At the 24-hr time point, the HIE + Vehicle group lost a significant amount of weight when compared with the Sham group. The HIE + 0.3  $\mu$ g IFN $\beta$  was not significantly different from the Sham group (\*p < .05 vs. Sham; Figure 4(c) and (d)). At the 48-hr time period, both the IFN $\beta$  treatment and Vehicle were significantly different from the Sham group (#p < .05 vs. Sham). Significance determined based on comparisons to the Sham 24-hr group and the Sham 48-hr group (\*p < .05 vs. Sham- 24 hr; #p < .05 vs. Sham- 48 hr) as calculated by Tukey's test. Also at the 24-hr time point, the decrease in weight loss effect of IFN $\beta$  treatment was reversed in the WP1066 + 0.3  $\mu$ g IFN $\beta$  (N = 5 per group). Significance determined based on comparisons to the Sham and 0.3  $\mu$ g IFN $\beta$  (\*p < .05 vs. Sham; #p < .05 vs. 0.3  $\mu$ g IFN $\beta$ ) as calculated by Tukey's test.



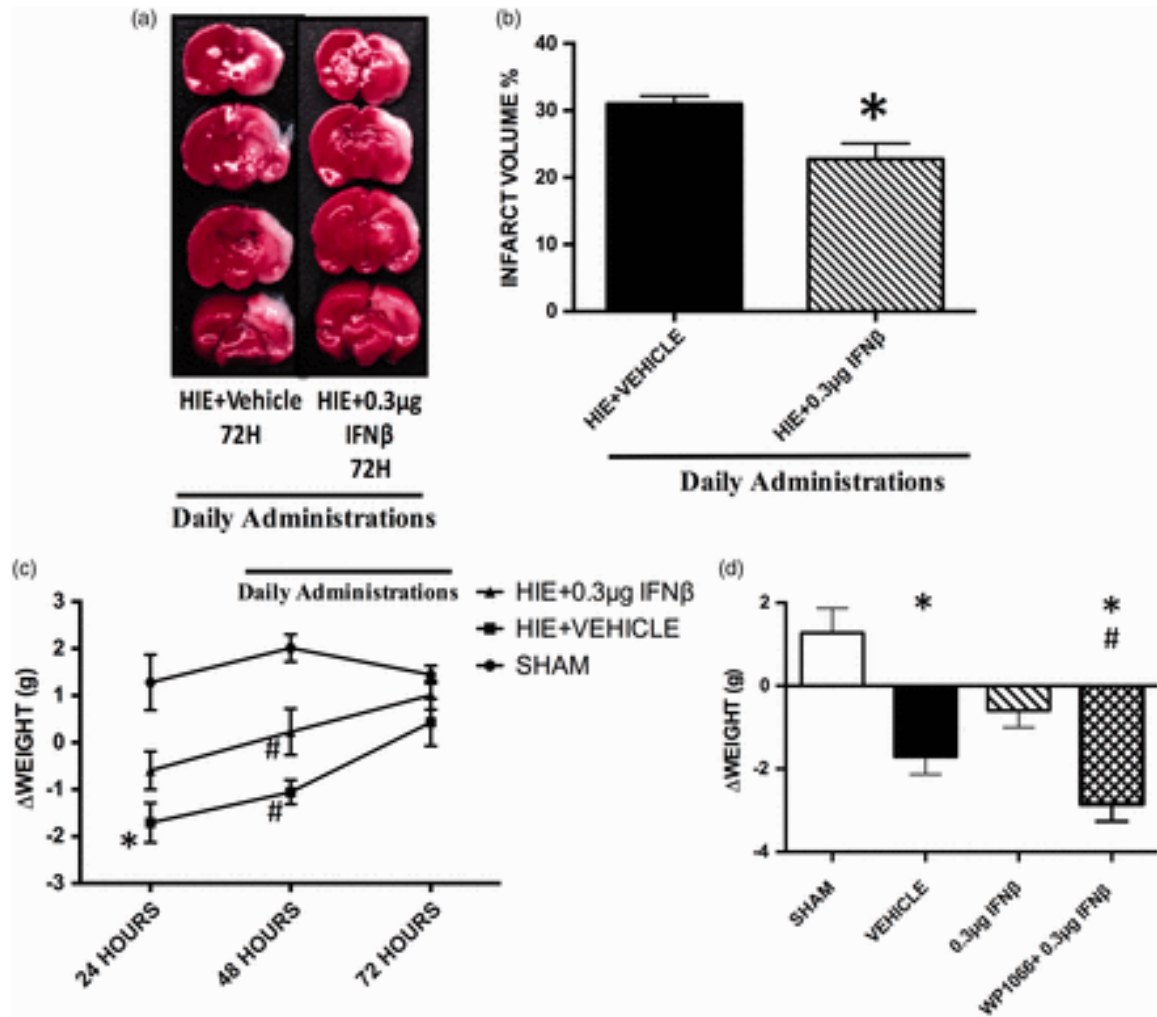


Figure 8: The effects of daily interferon beta administration after 72 hr. (a) Illustrates TTC staining of 2 mm brain slices 72 hr after hypoxic-ischemic encephalopathy. (b) Displays that intranasal 0.3  $\mu$ g IFN $\beta$  decreases infarct size 72 hr after injury. Significance determined based on comparisons to the HIE+Vehicle group (\*p < .05 vs. HIE+Vehicle) as calculated by student's T test. (c) A time course of changes in weight after hypoxic-ischemic encephalopathy. Significance determined based on comparisons to the Sham 24-hr group and the Sham 48-hr group (\*p < .05 vs. Sham- 24 hr; #p < .05 vs. Sham- 48 hr) as calculated by Tukey's test. (d) The effects of IFN $\beta$  treatment on weight at 24 hr. Significance determined based on comparisons to the Sham and 0.3  $\mu$ g IFN $\beta$  (\*p < .05 vs. Sham; #p < .05 vs. 0.3  $\mu$ g IFN $\beta$ ) as calculated by Tukey's test. HIE = hypoxic-ischemic encephalopathy; IFN $\beta$  = interferon beta.

Figure 5 displays data acquired from short-term neurobehavioral tests up to the 72-hr time point. The righting reflex test, a motor functioning test, shows that the HIE + Vehicle group (N = 5 rats per group) required a significantly longer time to correct their positioning when compared with the Sham groups (N = 7 rats per group) indicating physical dysfunction at 24 hr. The HIE + Vehicle was also significantly increased at the 72-hr time point as well. Interferon treatment was able to decrease righting reflex time and was not significantly different from the Sham group. The protective effect of IFN $\beta$  was diminished in the HIE + WP1066 + 0.3  $\mu$ g IFN $\beta$  group (N = 5 rats per group; Figure 5(c)). Significance determined based on comparisons to the Sham group (\*p < .05 vs. Sham) as calculated by Tukey's test. The geotactic reflex test illustrates similar results to the righting reflex test (Figure 5(b)). Animals that received IFN $\beta$  intranasal treatments were able to recognize that they were on an inclined surface and turned 180° faster, when compared with the vehicle group. Furthermore, the effects of IFN $\beta$  were reversed, when WP1066 was used in combination with IFN $\beta$  treatment as well (N = 5 rats per group; \*p < .05 vs. Sham; #p < .05 vs. Vehicle; Figure 5(d)). Significance determined based on comparisons to the Sham and Vehicle groups (\*p < .05 vs. Sham; #p < .05 vs. Vehicle) as calculated by Tukey's test.

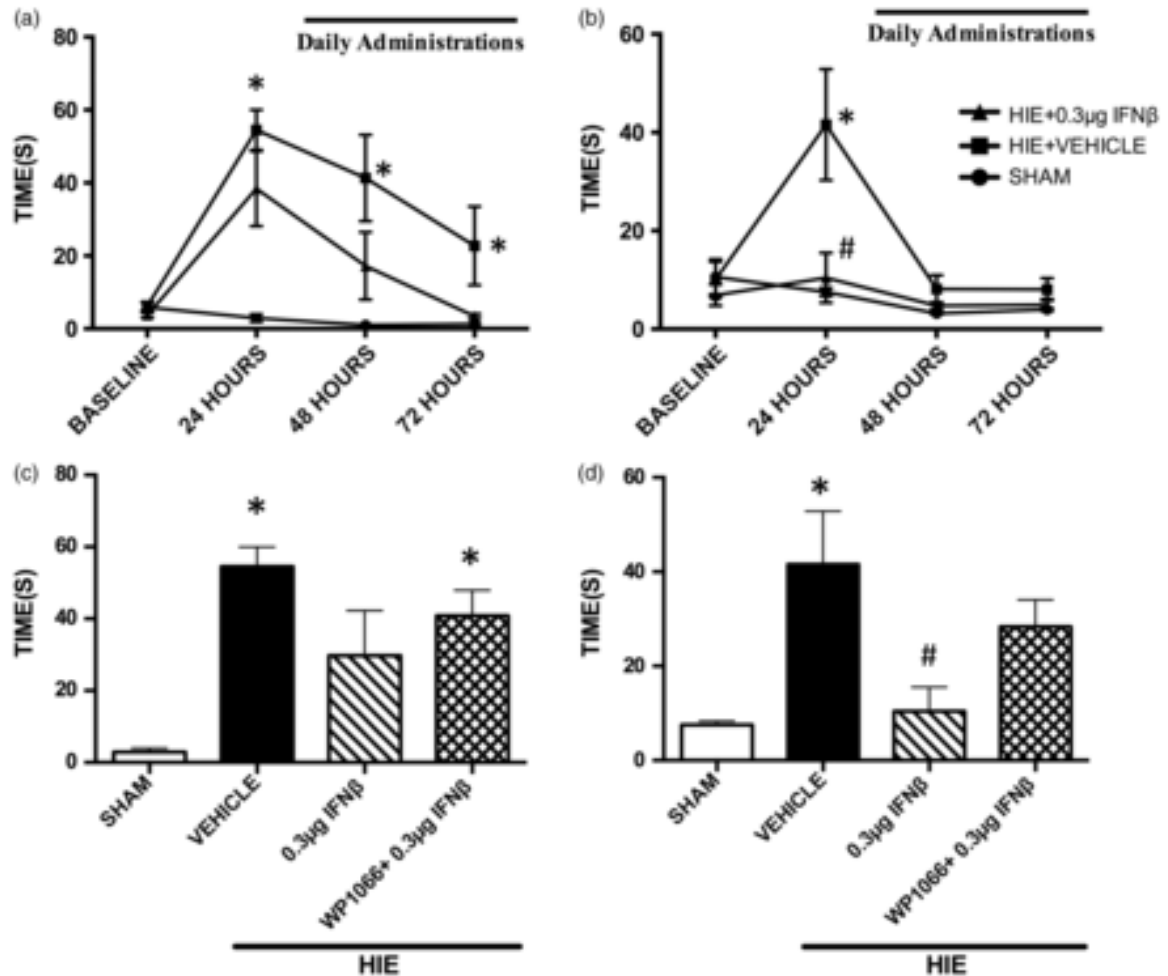


Figure 9: Short-term neurobehavioral tests after hypoxic-ischemic encephalopathy. (a) A time course of righting reflex behavior times after hypoxic-ischemic encephalopathy. (b) A time course of geotactic neurobehavior times after hypoxic-ischemic encephalopathy after 72 hr. (c) Intranasal treatment of 0.3 µg IFNβ improved righting reflex times at the 24-hr time point. Significance determined based on comparisons to the Sham group (\* $p < .05$  vs. Sham) as calculated by Tukey's test. (d) Intranasal treatment of 0.3 µg IFNβ improved geotactic reflex times at the 24-hr time point. Significance determined based on comparisons to the Sham and Vehicle groups (\* $p < .05$  vs. Sham; # $p < .05$  vs. Vehicle) as calculated by Tukey's test. HIE = hypoxic-ischemic encephalopathy.

***Intranasal Administration of IFN $\beta$  Increases Anti-Apoptotic Proteins and Decreases Fluoro-Jade Positive Neurons***

Intranasal IFN $\beta$  significantly increased expression of anti-apoptotic proteins and decreased neuronal cell death (Figures 6 and 7). P-STAT3 expression was increased in the HIE + 0.3  $\mu$ g IFN $\beta$  group when compared with the HIE + Vehicle group 24 hr post injury. Also P-STAT3 expression was significantly decreased after inhibition with WP1066 (N = 5 per group; Figure 6(b)). Significance determined based on comparisons to the HIE + Vehicle, HIE + 0.3  $\mu$ g IFN $\beta$ , and Sham groups (\*p < .05 vs. HIE + Vehicle; #p < .05 vs. HIE + 0.3  $\mu$ g IFN $\beta$ ; (p < .05 vs. Sham) as calculated by Tukey's test. Bcl-2 expression was significantly decreased after HIE. Intranasal IFN $\beta$  treatment and IFN $\beta$  treatment in combination with STAT3 inhibition were able to increase Bcl-2 expression after HIE when compared with the vehicle (Figure 6(c)). Significance determined based on comparisons to the Sham and HIE + Vehicle groups (\*p < .05 vs. Sham; #p < .05 vs. HIE + Vehicle) as calculated by Tukey's test. Cleaved caspase-3 expression was significantly increased in the HIE + Vehicle and HIE + WP1066 + 0.3  $\mu$ g IFN $\beta$  groups. Cleaved caspase-3 expression was lower in the HIE + 0.3  $\mu$ g IFN $\beta$  after 24 hr and not significantly different from any of the other groups (p = .0633 vs. Sham; N = 5 rats per group for all groups; Figure 6(d)). Significance determined based on comparisons to the Sham group (\*p < .05 vs. Sham) as calculated by Tukey's test.

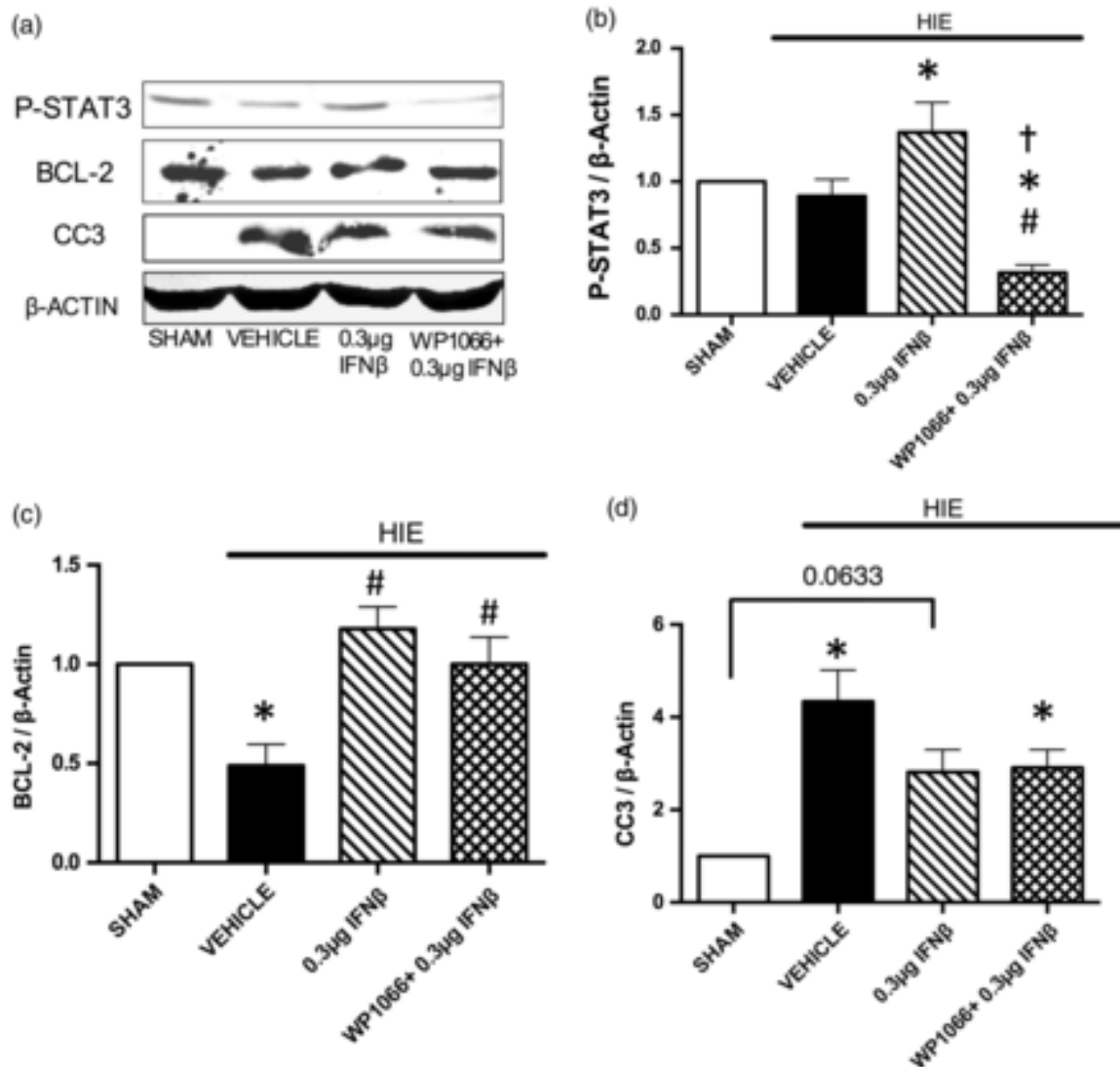


Figure 10: Expression of anti-apoptotic proteins involved with interferon beta treatment 24 hr after hypoxic-ischemic encephalopathy. (a) Representative Western blot images of P-STAT3, Bcl-2, and cleaved caspase-3 (CC3) expression in the injured right hemisphere of the brain 24 hr after hypoxic-ischemic encephalopathy. (b) Western blot analysis of P-STAT3 expression in the injured right brain hemisphere 24 hr after hypoxic-ischemic encephalopathy. Significance determined based on comparisons to the HIE+Vehicle, HIE+0.3  $\mu$ g IFN $\beta$ , and Sham groups (\*p < .05 vs. HIE+Vehicle; #p < .05 vs. HIE+0.3  $\mu$ g IFN $\beta$ ; †p < .05 vs. Sham) as calculated by Tukey's test. (c) Western blot analysis of BCL-2 expression in the injured right brain hemisphere 24 hr after injury. Significance determined based on comparisons to the Sham and HIE+Vehicle groups (\*p < .05 vs. Sham; #p < .05 vs. HIE+Vehicle) as calculated by Tukey's test. (d) Western blot analysis of cleaved caspase-3 expression in the injured right brain hemisphere at 24 hr after injury. Significance determined based on comparisons to the Sham group (\*p < .05 vs. Sham) as calculated by Tukey's test. HIE = hypoxic-ischemic encephalopathy.

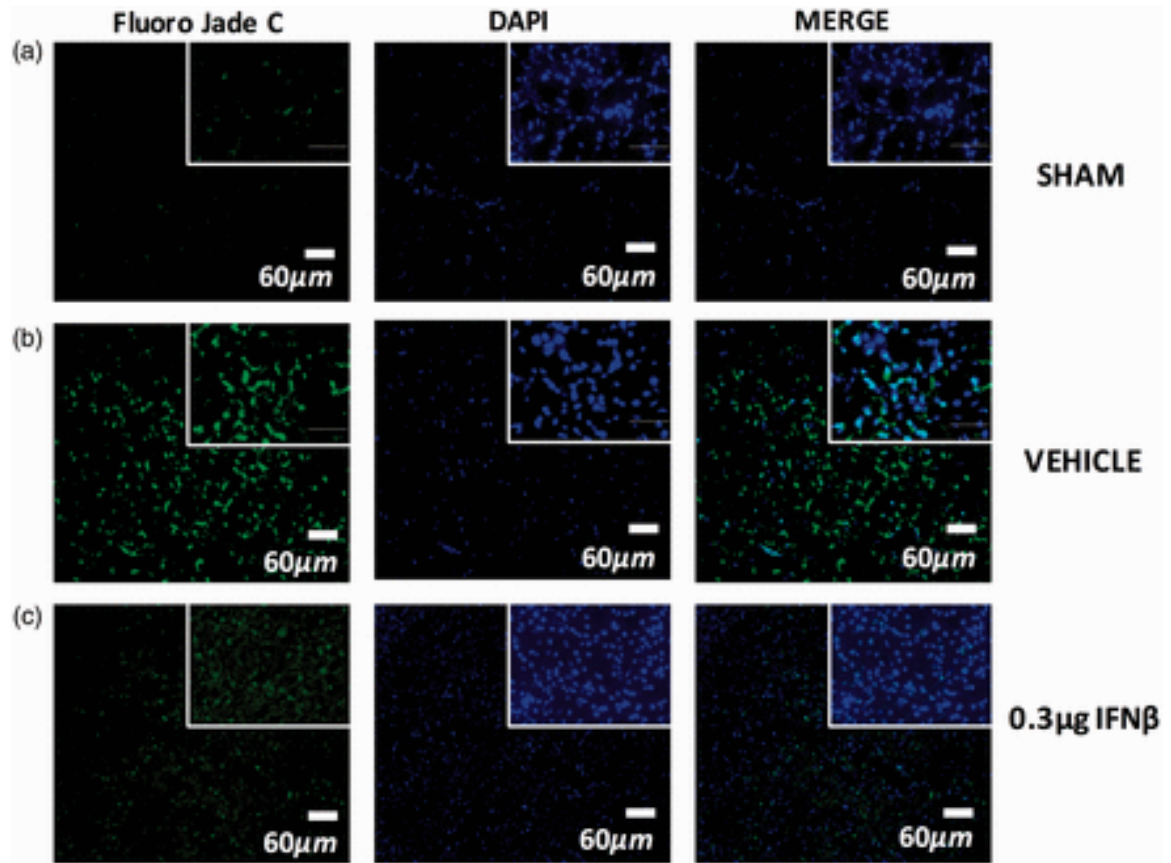


Figure 11: Illustrates positive Fluoro-Jade C expression in the central nervous system after 24 hr after injury. (a) Shows positive staining in the hippocampal region of the brain in the sham group at the 24-hr time point after utilizing immunohistochemistry and a Fluoro-Jade C kit. (b) Illustrates positive Fluoro-Jade C staining in the hippocampal region of the brain in the vehicle group after 24 hr using immunohistochemistry and a Fluoro-Jade C kit. (c) Immunohistochemistry and Fluoro-Jade C staining kit revealed positive Fluoro-Jade C staining in the hippocampal region of the brain after intranasal administration of 0.3 μg IFNβ. IFNβ = interferon beta.

After HIE, there was more positive Fluoro-Jade C expression in the hippocampal region in the HIE + Vehicle group. Intranasal IFN $\beta$  treatment decreased the expression of positive Fluoro-Jade C expression, the Sham group also had a decreased expression as well (Figure 7).

## **Discussion**

There are a few studies evaluating the effects of IFN $\beta$  treatment after ischemic stroke (Veldhuis et al., 2003; Marsh et al., 2009; Kuo et al., 2016). One of the earliest studies explored IFN $\beta$  treatment after middle cerebral artery occlusion in a rabbit model and found that IFN $\beta$  was neuroprotective and decreased infarct volumes (Liu et al., 2002). However, another study reported that IFN $\beta$  treatment failed following middle cerebral artery occlusion in a rat model. The authors of this study suggested that IFN $\beta$  treatment was unable to reach the affected tissues in the CNS since the blood–brain barrier was not sufficiently disrupted in their model (Maier et al., 2006).

Thus in our study, we chose intranasal administration of IFN $\beta$  treatment since it is clinically viable and noninvasive (Brown and Liu, 2014). Also intranasal delivery of IFN $\beta$  has previously been shown to effectively bypass the blood–brain barrier and directly target the CNS in a rat model of multiple sclerosis (Ross et al., 2004). To our knowledge, this is the first study to explore the effects of IFN $\beta$  treatment after neonatal HIE as well as the first to explore anti-apoptotic mechanisms involved with IFN $\beta$  in this model.

In the present study, we observed that overall expression of both the IFNR and IFN $\beta$  decreases in the CNS after neonatal HIE. We also found that intranasal IFN $\beta$

treatment was able to decrease infarction volumes after 24 hr and 72 hr, improve short-term neurobehavior at those time points, as well as improve postsurgery weight gain. Intranasal IFN $\beta$  treatment also had an anti-apoptotic effect of increasing Stat3 and Bcl-2 expression in addition to decreasing the expression of cleaved caspase-3 and neuronal degeneration. Moreover, pharmacological inhibition of Stat3 expression with the specific Stat3 inhibitor, WP1066, was able to reverse the protective effects of IFN $\beta$  that we observed. Pharmacological inhibition of Stat3 reversed protective effects of decreased infarction volumes and short-term behavior after HIE when applied in combination with intranasal IFN $\beta$  treatment.

The importance of Stat3 expression after stroke and neonatal HIE has been controversial. Our results show that IFN $\beta$  treatment increased Stat3 activation, the downstream product, after HIE. It has been previously suggested that treatments with the ability to activate Stat3 expression have been found to improve behavioral functions and decrease cell death after stroke (Raible et al., 2014). Our previous study demonstrating the effects of granulocyte-colony stimulating factor (G-CSF) in an experimental model of HIE observed that Stat3 was among some of the proteins contributing to anti-apoptosis in the study (Yata et al., 2007). Kinouchi et al. (2012) also found that activation of Stat3 in the peri-infarct region was associated with inhibition of apoptosis in a model of middle cerebral artery occlusion. Conversely, Hristova et al. observed that complete knockout of Stat3 in neurons and astrocytes after neonatal HIE in mice reduced brain damage and attenuated apoptosis by decreasing microglial activation, astroglial activation, and reactive astrogliosis. Interestingly, the same study also found that successful preinsult and



postinsult pharmacological inhibition of Stat3 with WP1066 resulted in a weak response that did not reduce brain tissue loss (Hristova et al., 2016).

Similarly, we observed that pharmacological Stat3 inhibition in combination with IFN $\beta$  treatment also did not yield improvement to brain infarction volume. Also Stat3 inhibition in concert with IFN $\beta$  treatment did not improve short-term neurobehavior or increases in anti-apoptotic protein expression suggesting that the beneficial effects of IFN $\beta$  treatment are mediated through Stat3 expression.

Our previous publications have demonstrated that hypoxic ischemic injury in the CNS induces the expression of Bax leading to apoptotic cell death (Charles et al., 2012). We have also previously demonstrated that the Bcl-2 is significantly decreased 24 hr after HIE and that increases in its expression leads to decreases in cleaved caspase-3 and neuronal cell death (Charles et al., 2012; Li, Klebe, et al., 2015). Other studies have also shown that increases in Bcl-2 lead to a decrease in neuronal apoptosis as well (Hu et al., 2014; Chen, Xu, et al., 2015). Similarly, our results show that Bcl-2 is decreased 24 hr after HIE and that intranasal IFN $\beta$  is able to increase Bcl-2 expression after injury contributing to neuronal survival. We believe that this effect is mediated by an increase in Bcl-2 which was induced by Stat3 activation via IFN $\beta$  treatment. Bcl-2 expression was also elevated in the Stat3 inhibition group as well. Since we observed the whole cellular expression of Bcl-2 in the injured hemisphere it is possible that other cell types contributed to the elevation of Bcl-2 in response to Stat3 inhibition.

It has been previously shown that cleaved caspase-3 expression increases after HIE and peaks at the 24-hr time point contributing to neuronal apoptosis (Wang et al., 2001; Askalan et al., 2015). Increased expression of positive Fluoro-Jade C staining has been

observed before after 24 hr after HIE (Aggarwal et al., 2014). Our studies also showed that cleaved caspase-3 expression increased after 24 hr in the HIE + Vehicle group and that there is increased positive staining of Fluoro-Jade C expression as well. In our studies, intranasal IFN $\beta$  treatment was able to decrease cleaved caspase-3 expression and neuronal apoptosis after 24 hr after injury.

Following HIE, sensorimotor functions and reflex maturation are severely affected as well (Sanches et al., 2012; Alexander et al., 2014). Our findings show that IFN $\beta$  treatment was able to improve sensorimotor deficits and reflex maturation delays since there was an improvement in the performance on the righting reflex and geotactic reflex short-term neurobehavioral tests after HIE. Body weights of the rat pups can also be used as an indicator of general health after HIE (Chen, Ma, et al., 2011), thus we recorded the weights of the rat pups each day after injury. Normally after HIE, there is a significant amount of loss in weight after injury as evidenced by the literature and our previous studies (Carty et al., 2008; Fathali et al., 2013). In the present study, our data show that IFN $\beta$  treatment was able to provide an improvement of general health since weight loss after injury was significantly reduced.

Our results also demonstrated that IFN $\beta$  treatment had no effect on brain edema and blood–brain barrier permeability at the 24-hr time point. This result helped us focus our attention upon the anti-apoptotic effects that IFN $\beta$  treatment had on neurons since we observed decreases in the infarction volume at the 24-hr time point. Our results also showed that there was no detectable blood–brain barrier disruption at 24 hr after neonatal HIE injury as the vehicle group is not significantly different from the sham control group. Previous studies of neonatal HIE from our laboratory and the literature confirm and

report similar findings that detectable disruptions of the blood–brain barrier occur at 48 hr after neonatal stroke in the rat pup model (Fernandez-Lopez et al., 2012; Li, McBride, et al., 2015).

In conclusion, we report that intranasal administration of human recombinant IFN $\beta$  treatment was able to attenuate neuronal apoptosis via the Jak1/Stat3/Bcl-2 pathway after neonatal HIE (Figure 8). We determined that intranasal human recombinant IFN $\beta$  treatment induces Stat3 activation and leads to increased expression of Bcl-2 which led to a decrease in cleaved caspase-3 expression and neuronal degeneration. IFN $\beta$  treatment has the potential to become a new therapeutic strategy after HIE, however more translational studies are still needed. Future studies should also explore potential anti-inflammatory mechanisms after IFN $\beta$  administration after HIE.

### **Summary**

Intranasal treatment with human recombinant IFN $\beta$  reduces brain injury and improves neurological functions after hypoxic-ischemic injury. These findings suggest that IFN $\beta$  could be a potential therapy for neonatal hypoxic-ischemic encephalopathy in the future.

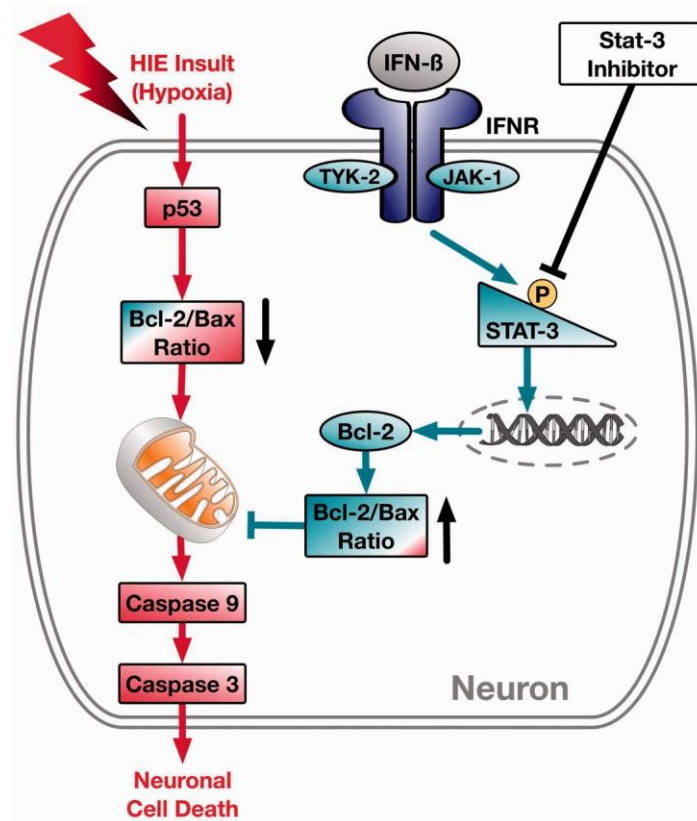


Figure 12: A visual schematic. In our experiments human recombinant interferon beta (IFN- $\beta$ ) administered through the intranasal route was able to bind and activate the Type 1 interferon receptor (IFNR). Activation of IFNR leads to increases of P-Stat3 activation and transcription of Bcl-2, which increases the Bcl-2/Bax ratio. This eventually leads to the decreased expression of caspase 3 and ultimately decreases in neuronal cell death. IFN $\beta$  = interferon beta; IFNR = Type 1 interferon receptor.

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**CHAPTER THREE**  
**ISOFLURANE PROVIDES NEUROPROTECTION IN NEONATAL HYPOXIC**  
**ISCHEMIC BRAIN INJURY**

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The work presented in this chapter has been published.  
J Investig Med. 2013 October; 61(7): 1078–1083

## **Abstract**

Isoflurane is a volatile anesthetic that is widely used clinically as an inhalational anesthetic. In recent years, several studies have indicated that isoflurane has neuroprotective properties. This has led to the beneficial effects of isoflurane being analyzed in both cell culture and animal models, including various models of brain injury. Neonatal hypoxia ischemia may be characterized as injury that occurs in the immature brain, resulting in delayed cell death via excitotoxicity and oxidative stress. These adverse events in the developing brain often lead to detrimental neurological defects in the future. Currently, there are no well-established effective therapies for neonatal hypoxia ischemia. In line with this, isoflurane, which displays neuroprotective properties in several paradigms and has been shown to improve neurological deficits caused by brain injuries, has the capability to be an extremely relevant clinical therapy for the resolution of deficits concomitant with neonatal hypoxic ischemic brain injuries. This review will therefore seek to explore and analyze the current information on isoflurane, looking at general isoflurane anesthetic properties, and the protection it confers in different animal models, focusing particularly on neuroprotection as shown in studies with neonatal hypoxic ischemic brain injury.

## **Isoflurane and Other Volatile Anesthetics**

Isoflurane is a volatile anesthetic that has been in clinical practice for several decades. Volatile anesthetics are also known as inhalational anesthetics and, apart from isoflurane, also include sevoflurane, desflurane, halothane, and enflurane. Of the five major volatile anesthetics, isoflurane, sevoflurane, and desflurane are most commonly used today. Halothane and enflurane are not used widely due to undesirable side effects<sup>1</sup>. The volatile anesthetics all differ in potency, adverse effects, and cost, and are used extensively during surgery in human neonates and during neonatal animal research. Moreover, volatile anesthetics are employed in about 80% of the surgeries in the United States that are performed under general anesthesia<sup>2</sup>. Volatile anesthetics have been reported to produce their effects by a number of mechanisms, including antagonism of ionic channels, activation of glycine and  $\gamma$ -aminobutyric acid (GABA) receptors, and alteration of the function and activity of other cellular proteins, as well as through utilizing high lipid solubility characteristics<sup>3,4</sup>. Isoflurane (2-chloro-2-(difluoromethoxy)-1,1,1-trifluoro-ethane,  $\text{CHF}_2\text{-O-CHCl-CF}_3$ ), has been shown to be more potent than several other anesthetics. It is much more widely used in comparison to the other inhalational fluorinated inhalational anesthetics<sup>5</sup>. Isoflurane was discovered in 1965 by Dr. Ross Terrell and associates in their quest for a better inhaled anesthetic than was available at the time and was the 469th compound found, following enflurane and halothane<sup>5</sup>. Isoflurane has a minimum alveolar concentration (MAC) of 1.15%, and blood/gas partition coefficient of 1.4, lower than that of most other inhaled anesthetics<sup>6</sup>. Also, isoflurane is very stable and is resistant to biodegradation<sup>3</sup>. These properties of

isoflurane allow it to rapidly induce unconsciousness and also to be quickly eliminated from circulation.

### **Isoflurane is Protective in Various Animal Models**

Isoflurane has been studied in animal models of various diseased states, such as Lipopolysaccharide (LPS)-induced Acute Inflammation of the Lung<sup>7</sup>, Acute Lung Injury<sup>8</sup>, glucose-induced oxidative stress<sup>9</sup>, renal ischemia/reperfusion injury<sup>10,11</sup>, cardiac injury<sup>12</sup>, etc. In these models, it is shown to provide protection from injury and improve various negative functional outcomes. The protective effects of isoflurane in these models are reported to occur through the activation of several pathways, and are briefly outlined in Table 1.

Table 3. Isoflurane shows protection in various animal models

Animal Model	Isoflurane Protection	Source
<b>General Models</b>		
LPS-Induced Acute Lung Inflammation (Rat)	Inhibited ROS burst, NF- $\kappa$ B activation, and pro-inflammatory cytokines	Chung, I.S. et.al. 2013
T/HS-induced ALI	Inhibited platelets through the ADP pathway	Harr, N. et.al. 2012
Renal Ischemia/ Reperfusion Injury	Reduced renal dysfunction and tubular necrosis via the SK/S1P pathway	Kim, M. et.al.2007; Lee, H.T. et.al. 2004
Cardiac Injury	Attenuated the release of LDH and CK-MB, and reduced infarct size via PKC $\epsilon$ /ALDH2 pathway	Lang, X. et.al. 2013
High Glucose-induced Oxidative Stress in Isolated Human Artery	Preserved vascular function by restoring ATP-sensitive K <sup>+</sup> channel function	Kinoshita, H. et.al. 2012
<b>Stroke Models</b>		
Subarachnoid Hemorrhage	Prevented post SAH neuronal apoptosis and BBB disruption in ipsilateral hemisphere through S1P pathway	Altay, O. et.al. 2012
Middle Cerebral Artery Occlusion	Improved neurological outcome, and reduced inflammatory cytokine production via attenuation of NF- $\kappa$ B activation	Li, H. et.al. 2013
Intracerebral Hemorrhage	Reduced brain edema, apoptotic cell death, and neurological deficits	Khatibi, N. H. et.al. 2011
Traumatic Brain Injury	Increased CA3 neuronal survival, and improved histopathology	Statler, K.D. et.al. 2006
Neonatal Hypoxic Ischemic Brain Injury	Reduced infarction volume, improved neurobehavioral outcome, and increased pAkt	Zhou, Y. et.al. 2010

In particular, isoflurane shows neuroprotection in several stroke models including subarachnoid hemorrhage (SAH)<sup>13</sup>, middle cerebral artery occlusion (MCAO)<sup>14,15</sup>, intracerebral hemorrhage<sup>16</sup>, traumatic brain injury<sup>17</sup>, and Neonatal Hypoxic Ischemic Brain Injury (HI)<sup>18</sup> (Table 1). For this review, the main focus will be on the neuroprotection of isoflurane as studied in neonatal HI, its possible mechanisms of action, and what direction this could be leading us in terms of clinical applications of isoflurane treatment.

### **Neonatal Hypoxia-Ischemia**

Neonatal Hypoxia Ischemia is a major cause of mortality and neurological deficits such as cerebral palsy, mental retardation, and epilepsy in the perinatal period<sup>19,20</sup>. Several factors have been implicated in the pathophysiology of HI, including inflammatory mediators, excitotoxicity, and oxidative stress<sup>21</sup>. While extensive research has been done on this form of brain injury, there is a gap between the basic and clinical sciences as successful treatments are still lacking. There is still a significant number of neonates who suffer from perinatal HI and its repercussions, approximately 1 in 4000, a figure two to three times higher than the incidence of childhood stroke<sup>22</sup>. The methods of interventions are often limited for neonatal HI, since neuronal cell death is frequently only delayed, although it usually appears to be prevented shortly after an assault. Thus, it is essential that more preclinical studies be carried out to elucidate the possibility of an effective therapy that will provide long-term neuroprotection against HI in the perinatal period, reducing the effects seen up to adulthood. To study neonatal hypoxic ischemic brain injury, the Rice-Vannucci model is the one that is most widely used. Induction is

most commonly with isoflurane at 3% and occurs for 4 minutes, followed by ligation of the right common carotid artery in rat pups. Isoflurane is continuously infused for the duration of the surgery (typically not longer than 5 minutes) at 2.5%. The inspired concentration of isoflurane is lowered (from 3% to 2.5%) as this compensates for the decreased uptake due to the equilibration of isoflurane in the body following the induction period<sup>5</sup>. The pups are then subjected to a hypoxic chamber with 8% oxygen at 37°C for 2.5 hours. Infarction volumes are calculated and other neurological assessments are done at different time points after surgery. This method has been modified from that described by Rice, Vannucci, and Brierly, and is most commonly done in postnatal day 7 or 10 rats<sup>23</sup>. P7 rat pups represent a human fetus at gestational age 32 to 34 weeks or a newborn infant at this stage<sup>24</sup>, while P10 rats are similar to the older neonate approaching adolescent stages of development or a full-term neonate<sup>25</sup>.

### **Neuroprotection of Isoflurane in Neonatal HI: Possible Mechanisms**

The use of isoflurane as an anesthetic in the surgery for modeling neonatal HI, along with the great variations observed in infarct sizes within and among litters, particularly as surgery times varied has led to speculations on the effects that isoflurane may be having on these neonates. This, among other reasons, has therefore sparked the interest in studying whether or not isoflurane may be providing some form of neuroprotection against ischemic injury in neonatal rat pups. As a result, several studies have found that isoflurane, when it is administered before or after an experimental hypoxic-ischemic insult (pre- and post-conditioning) in neonates, has the ability to reduce



negative outcomes. Studies indicate that isoflurane acts through a number of mechanisms that are involved with neuroprotection and an increase in neuronal viability (Figure 1).

It has been suggested that isoflurane inhibits excitotoxicity that is initiated by an accumulation of glutamate during ischemia, resulting in a reduction in necrosis. The antagonism of glutamate receptors by isoflurane has been shown in in vitro studies, as well as in in vivo models where isoflurane reduced extracellular glutamate during ischemia<sup>26</sup>. In addition, isoflurane has been shown to be a GABA receptor agonist, providing an inhibitory effect against excitotoxicity<sup>3</sup>. Under hypoxic conditions isoflurane was shown to be protective via its interaction with the pathways that involve phospholipase C and the release of Ca<sup>2+</sup> from intracellular stores. Phospholipase C triggers the Phosphatidylinositol-3-kinase/Protein Kinase B and Mitogenactivated protein kinase (PI3K/Akt/MAPK) pathways, which are anti-apoptotic<sup>27</sup>. Isoflurane also induced neuroprotection by altering the phosphorylation states of players in both the calcium-dependent and calcium-independent MAP kinase pathways, increasing the phosphorylation of some of the kinases (Pyk-2, ERK, MKK-6, JNK), while decreasing the phosphorylation of the downstream MAP kinase dependent transcription factors (p38, pElk-1 p90 RSK, ATF-2). Phosphorylation of the MAP kinases was prevented by an inositol triphosphate (IP3) receptor antagonist, suggesting that this phenomenon after isoflurane preconditioning depends on the small increases in intracellular Ca<sup>2+</sup>. The study also showed that isoflurane enabled neurons to avoid potentially toxic levels of Ca<sup>2+</sup> increase. Moreover, there was an observed increase in the levels of Akt, an anti-apoptotic protein, due to isoflurane preconditioning<sup>27,28</sup>. The activation of the Akt pathway was also observed in a model of oxidative and inflammatory stress on cardiac myocytes.

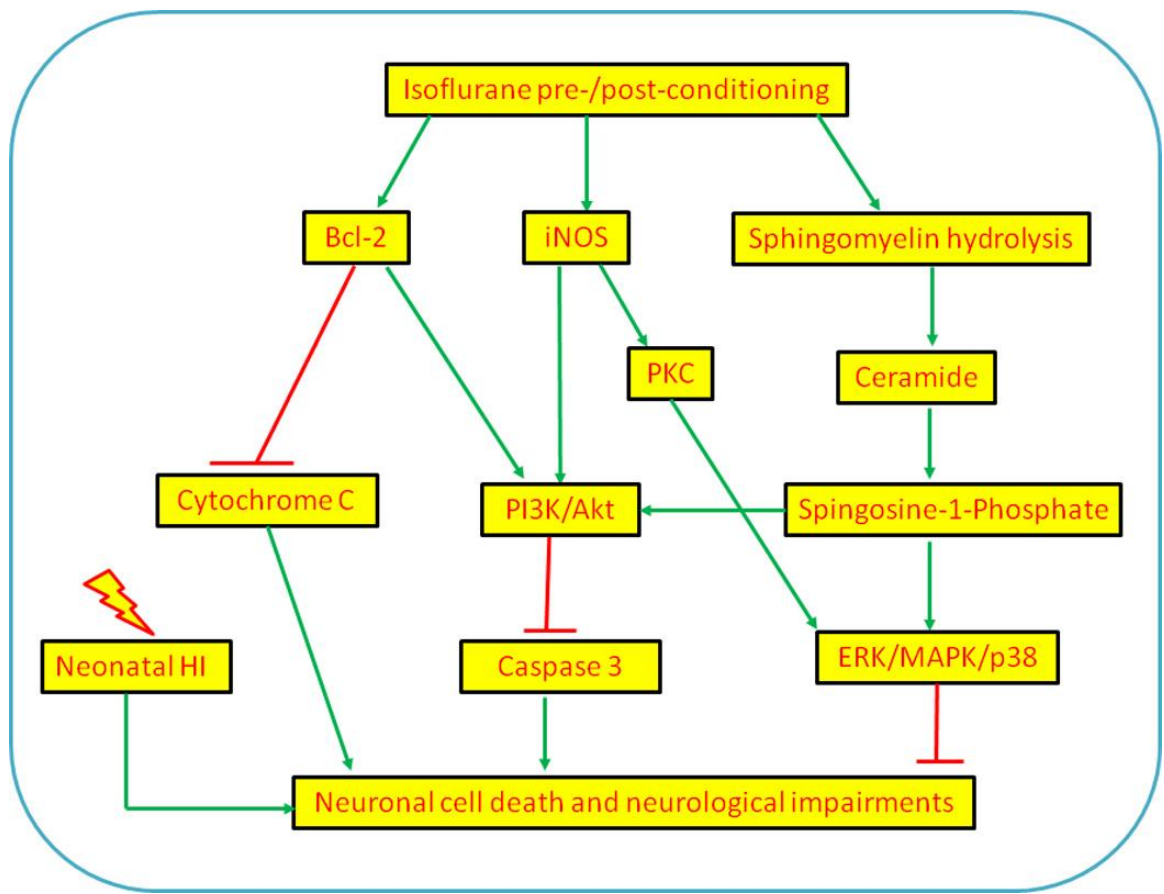


Figure 13. Proposed Mechanisms of Isoflurane Neuroprotection. Other potential mechanisms have been explored; however, these are the most extensively addressed in this review<sup>18,29,40,41</sup>.

Isoflurane was shown to prevent apoptosis by activating Akt and enhancing B-cell lymphoma-2 (Bcl-2) expression<sup>29,30</sup>. Furthermore, isoflurane was shown to protect against oxygen glucose deprivation-induced ischemia in neuronal cultures by activating the cell-surviving protein, hypoxia inducible factor -1 $\alpha$  (HIF-1 $\alpha$ ) and the subsequent increase in expression of inducible nitric oxide synthase (iNOS) mRNA. Notably, the protection observed with isoflurane preconditioning was also dependent on the extracellular signal-related kinase (ERK) pathway; an ERK1/2 inhibitor partially reversed the protective effects that resulted from isoflurane administration<sup>31</sup>. The aforementioned studies were mainly carried out in in vitro models of hypoxia, and though they may provide clearer mechanistic pathway descriptions, they differ from the physiology seen in intact animals. However, similar neuroprotection has been observed in vivo. In one study done in an animal model of neonatal HI, Chen et.al, hypothesized that longer isoflurane exposure times may be one of the underlying factors leading to the inconsistent results in infarction volumes observed in the model. The results showed that longer surgery times, and hence longer isoflurane exposure, resulted in decreased infarction severity in the rat pups. Pups that had exposure times to isoflurane of 13 or 21 minutes, had significantly less volume of infarction than those with only 5 minutes of exposure: 33.2% in the 5 minute group, versus 20.3% in the 13 minute group and 11.3% in the 21 minute group in the 7-day old pups. This trend was also observed in the 10-day old rat pups (24.8% in the 5-minute group versus 11.9% in the 13-minute group and 9.3% in the 21-minute group)<sup>25</sup>. By extension, this group also did experiments to elucidate the mechanism by which isoflurane was participating in this neuroprotective role. They found that the sphingosine-1-kinase/PI3K/Akt (S1P/PI3K/Akt) pathway is active in this function as administration

of selective inhibitors of S1P (VPC23019) and PI3K (Wortmannin) completely reversed the effects of isoflurane-induced neuroprotection. Importantly, isoflurane provided both short and long-term neuroprotection as effects were observed up to 4 weeks after the HI injury, when neurobehavioral tests were carried out. The effect of the S1P and PI3K inhibitors had a long-term effect as well, reversing the improvement in neurobehavior induced by longer exposures to isoflurane. Also, Akt phosphorylation was preserved in the isoflurane pretreated group, and a significant reduction in cleaved caspase-3 levels was observed. These findings confirmed the speculation of the involvement of the S1P/PI3K/Akt pathway<sup>18</sup>. In addition, in a SAH model, isoflurane was also shown to be neuroprotective by activating the sphingosine kinase/S1P (SK/S1P) pathway. Isoflurane treatment attenuated the disruption of the blood-brain-barrier (BBB) that usually occurs after SAH<sup>13</sup>. The involvement of the S1P/PI3K/Akt pathway in protection has also been implicated by other groups in different models of ischemia. Isoflurane was found to protect against renal dysfunction, significantly lowering plasma creatinine values and tubular necrosis, an effect abrogated by SK and S1P receptor antagonists, providing further evidence that their activity is essential to isoflurane-induced protection against ischemia. Additionally, mice lacking the SK1 enzyme were not protected against injury from ischemia<sup>10</sup>. The S1P pathway involvement in protection was seen in cardiac ischemia reperfusion injury also as the administration of S1P, which activated the S1P receptor, resulted in reduced infarct sizes, leukocyte recruitment and adhesion<sup>32</sup>. In models of liver ischemia, the SK/SIP pathway was also shown to be activated. There was a significant improvement in hepatic function in both normal and cirrhotic livers, increased anti-inflammatory cytokine production, and downregulation of anti-apoptotic

genes following ischemia-reperfusion injury due to the activation of S1P<sup>33</sup>. Volatile anesthetics, including isoflurane, have been proposed to exhibit potency as a result of their high lipid solubility<sup>3</sup>. This theory was proposed by Meyer and Overton in 1901 and has been used to explain the involvement of the lysophospholipid, Sphingosine-1-phosphate, which results from the activation of sphingomyelin hydrolysis<sup>34</sup>. Sphingomyelin is a lipid component of the membrane which is hydrolyzed to ceramide, a precursor of sphingosine. Sphingosine is then converted to S1P by the enzyme sphingosine kinase<sup>10</sup>. Sphingosine kinase is involved in many different biological processes including cell growth and cell survival<sup>35</sup>. In addition, sphingosine kinase and S1P have been shown to upregulate the ERK/MAPK pathway, resulting in increased cell survival<sup>36,37</sup>. A significant finding was that animals with a knockout of the SK gene could not be protected by isoflurane treatment<sup>10,38</sup>. These studies all correlate with the Lipid Theory which describes the potency of volatile anesthetics in relation to lipid solubility. However, in recent years, the Protein Theory, which postulates that anesthetics act directly on proteins rather than accumulating in membranes, has stimulated a lot of interest<sup>3,39</sup>. Consequently, other mechanisms have been proposed on how isoflurane confers neuroprotection. One such pathway is through the activation of inducible nitric oxide synthases (iNOS). Zhao, et.al found that isoflurane induced an increase in nitric oxide and suggested that this may be the mediator for the neuroprotection induced by isoflurane. They confirmed this by the use of a nitric oxide inhibitor, aminoguanidine, which abolished the neuroprotection conferred by isoflurane<sup>22</sup>. Isoflurane neuroprotection was shown using a preconditioning model where isoflurane was given at 1.5% for 30 minutes 24 hours before the hypoxic insult. Neuroprotection was measured up to 7 days

post injury. In another study by the same group, it was found that the brain loss induced by HI was significantly attenuated up to one month following injury, when animals were preconditioned with 1.5% isoflurane for 30 minutes 24 hours before injury. This conclusion was made as there were no significant differences between control and isoflurane treated groups in long-term reference memory, as well as short-term working memory. Isoflurane also significantly improved motor coordination one month after injury. Additionally, preconditioning with isoflurane resulted in increased Bcl-2 expression in the hippocampus<sup>40</sup>. Bcl-2 can decrease the release of cytochrome C and mitochondrial membrane permeability caused by ischemic episodes<sup>40</sup>. Again, it was found that the protection by isoflurane could be reversed by administration of an inducible nitric oxide synthase inhibitor, suggesting a role for iNOS in the isoflurane-induced neuroprotection. Small increases in iNOS protein expression have been associated with cardioprotection and neuroprotection. iNOS is believed to be an important factor for protection since protein kinase C(PKC) and MAP kinase are both located downstream of iNOS and are believed to provide protection<sup>41</sup>. Notably, isoflurane was found to increase the p38 MAPK phosphorylation status after an ischemic injury and the reduction induced by preconditioning with isoflurane was abolished by the administration of a p38 MAPK inhibitor<sup>42</sup>. Kapinya et.al. also found that isoflurane preconditioning resulted in an increase in iNOS in an MCAO model and suggested that the increase in iNOS may be the means by which isoflurane reduces infarction volume and other neurological deficits. However, they also stated that further study is needed to identify the underlying anesthetic mechanism as the increase in iNOS was not observed until 6 hours after the preconditioning event, while protection was seen immediately after

preconditioning with isoflurane<sup>43</sup>. Nitric oxide is known to interact with two survival pathways: the Ras/Raf/MEK/ERK pathway and the PI3K/Akt pathway<sup>44</sup>. Both of these pathways are essential to neuronal survival and could therefore be the underlying mechanism by which the isoflurane-induced increase in iNOS is protective in brain injury models. However, there is some contradiction in this area as NO has been shown to be both protective and toxic<sup>44</sup>. Therefore, there are still further studies that need to be done to clarify the neuroprotection of isoflurane via an increase in iNOS. The studies described above show a long-term neuroprotective role of the anesthetic, isoflurane. This is significant as a large percentage of infants who suffer from neonatal HI do survive into adulthood; however, most of them have residual motor and cognitive deficits<sup>21</sup>. However, importantly, there have been contradictions on whether or not the neuroprotection provided by isoflurane is long-term, or only transient. A study done by McAuliffe, et.al, suggested that isoflurane may be useful in preventing the deficits seen long after the neonatal injury, or at least reducing the severity. This conclusion was made from experiments that showed that preconditioning with isoflurane improved striatal function in adulthood. Neurobehavioral testing was also done by the use of a water maze with adult mice that had undergone neonatal HI, and was done at 70 days old. Mice preconditioned with isoflurane performed as well as sham in this neurobehavioral test and this therefore provides evidence for long-term neuroprotection<sup>45</sup>. By contrast, Sasaoka, et.al found that preconditioning with isoflurane at 2% for either 60 or 90 minutes before the HI insult induced ischemic tolerance in the hippocampus of P7 rat pups up to 7 days after the injury, but not at 49 days<sup>20</sup>. These results suggest that the neuroprotective effect of isoflurane is only transient. This may be due to the rapid removal of isoflurane from

the system as a result of small amounts of biodegradation, as stated before. It has also been suggested that isoflurane preconditioning may simply be delaying neuronal injury, not preventing it. This idea was proposed based on results that showed that brain infarction in the isoflurane treated group was significantly reduced in comparison to controls two days following ischemic injury, but this reduction was not seen 14 days after the insult. However, isoflurane did reduce the number of necrotic cells in the penumbral area two weeks after ischemia was induced<sup>46</sup>. This experiment was carried out in an adult model of ischemia. Nevertheless, it still presents a significant consideration to be made with regards to the use of isoflurane for neuroprotection clinically because the delay in neuronal injury provided by isoflurane may provide a longer therapeutic window for other interventions.

### **Future Directions**

It is clear that isoflurane exhibits neuroprotective properties, and harnessing this capability of the volatile anesthetic may be very relevant clinically. However, while isoflurane has been shown to be beneficial in numerous cases, before it can be used as a neuroprotective therapy for neonatal HI, several concerns have to be addressed. One such consideration is the length of time that isoflurane is administered. Studies have shown that isoflurane exposure for periods of 3 to 6 hours resulted in caspase activation and neuronal death<sup>47,48</sup>, in contrast to the potent neuroprotection observed with shorter exposure times<sup>25,40</sup>. Therefore, studies specifically aimed at establishing an optimal time period within which there is still effective neuroprotection from isoflurane, without causing neurotoxicity, have to be done. There are contradictory evidences in support of



isoflurane playing a neuroprotective role, while at the same time, potentially being neurotoxic. As discussed above, there are many animal studies that show isoflurane to be convincingly neuroprotective. However, evidence based on clinical studies in humans is not as strong<sup>4</sup>. And while there is not much evidence on the neurotoxicity resulting from isoflurane anesthesia, the fact that there is any, is reason for considerations to be made in this regard before clinical isoflurane therapy can progress substantially. Isoflurane studies are clinically relevant and translatable because the percentage of isoflurane used experimentally is around 1.15% to 2%, which is comparable to a MAC of 1.15% in human adults and 1.6% in human neonates<sup>40</sup>. However, different researchers have found the most effective dosages to be different in their models. With these discrepancies, much more would need to be done to establish what the optimal dosage of isoflurane is that will be most effective, providing maximal neuroprotection to infants. Another factor that is very important to consider is elucidating the exact mechanism by which isoflurane provides protection from cell death and other negative outcomes. Several different pathways have been implicated to date. It was first postulated that isoflurane, and volatile anesthetics by extension, were more potent the more lipid-solubility they exhibited<sup>3</sup>. Since the membrane is composed mainly of lipids, they were thought to act through membrane lipids. Hence, sphingosine and sphingosine kinase were suggested to be involved. From this theory, several studies carried out in various models have shown that isoflurane provides neuroprotection by activating this pathway, which then upregulates the ERK/ MAPK/Akt pathway, resulting in increased cell growth and survival. However, newer theories indicate a more protein-based mechanism of action, via several ion channels, as well as GABA and glutamate receptors. The involvement of iNOS in

isoflurane-mediated neuroprotection is another popular mechanism proposed and is more in line with the Protein Theory. Notably, many of these pathways, including sphingosine-1-phosphate and iNOS, exhibit protection via the activation of the ERK/MAPK/Akt pathway. This is significant and may be the way to connect the different pathways implicated, thereby establishing how isoflurane is protecting neurons from cell death. Finally, neonatal HI results from several different pathophysiological manifestations. Hence, it may be necessary to target more than one of these to provide sufficient neuroprotection. Consequently, using isoflurane along with another intervention may be much more effective. Moreover, with the suggestion by some studies that isoflurane is merely delaying repercussions from ischemia, rather than preventing them, this may open a longer therapeutic window for other interventions to be applied, and may also provide an additive protective effect. The possibility of using isoflurane clinically is very promising, and with the fact that it is already so widely used, addressing the current concerns would prove very beneficial in progressing not only the field of neonatal brain injury, but also many diseases related to hypoxia and ischemia.

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## **CHAPTER FOUR**

### **G-CSF ATTENUATES NEUROINFLAMMATION AND STABILIZES THE BLOOD–BRAIN BARRIER VIA THE PI3K/AKT/GSK-3B SIGNALING PATHWAY FOLLOWING NEONATAL HYPOXIA-ISCHEMIA IN RATS**

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The work presented in this chapter has been published.  
Experimental Neurology 272 (2015) 135–144

## Abstract

*Objective:* Neonatal hypoxia occurs in approximately 60% of premature births and is associated with a multitude of neurological disorders. While various treatments have been developed, translating them from bench to bedside has been limited. We previously showed G-CSF administration was neuroprotective in a neonatal hypoxia-ischemia rat pup model, leading us to hypothesize that G-CSF inactivation of GSK-3 $\beta$  via the PI3K/Akt pathway may attenuate neuroinflammation and stabilize the blood–brain barrier (BBB).

*Methods:* P10 Sprague–Dawley rat pups were subjected to unilateral carotid artery ligation followed by hypoxia for 2.5 h. We assessed inflammation by measuring expression levels of IKK $\beta$ , NF- $\kappa$ B, TNF- $\alpha$ , IL-1 $\beta$ , IL-10, and IL-12 as well as neutrophil infiltration. BBB stabilization was evaluated by measuring Evans blue extravasation, and Western blot analysis of Claudin-3, Claudin-5, ICAM-1, and VCAM-1.

*Measurements and main results:* First, the time course study showed that p- $\beta$ -catenin/ $\beta$ -catenin, IKK $\beta$ , and NF- $\kappa$ B expression levels peaked at 48 h post-HI. The knockdown of GSK-3 $\beta$  with siRNA prevented the HI-induced increase of p- $\beta$ -catenin/ $\beta$ -catenin, IKK $\beta$ , and NF- $\kappa$ B expression levels 48 h after HI. G-CSF treatment reduced brain water content and neuroinflammation by downregulating IKK $\beta$ , NF- $\kappa$ B, TNF- $\alpha$ , IL-1 $\beta$ , and IL-12 and upregulating IL-10, thereby reducing neutrophil infiltration. Additionally, G-CSF stabilizes the BBB by downregulating VCAM-1 and ICAM-1, as well as upregulating Claudins 3 and 5 in endothelial cells. G-CSFR knockdown by siRNA and Akt inhibition by Wortmannin reversed G-CSF's neuroprotective effects.

*Conclusions:* We demonstrate G-CSF plays a pivotal role in attenuating



neuroinflammation and BBB disruption following HI by inactivating GSK-3 $\beta$  through the PI3K/Akt pathway.

## Introduction

Neonatal hypoxia-ischemia (HI) is an injury that occurs in less than 1% of full-term infant births; however, the occurrence of HI is increased to approximately 60% of premature births (Vannucci and Vannucci, 1997 and Volpe, 2001). In 20–40% of HI cases, infants develop neurological disorders, such as mental retardation, epilepsy, and cerebral palsy (Vannucci et al., 1999). Various experimental therapies have been investigated for improving HI patient outcomes and quality of life, yet successful clinical translation has been limited (Burchell et al., 2013, Koenigsberger, 2000 and Zanelli et al., 2009). Thus, it is of the utmost importance to identify novel therapeutics with potential for clinical translation.

Granulocyte stimulating factor (G-CSF) is a hematopoietic growth factor with known neuroprotective effects in animals models of ischemia (Liu et al., 2014a, Popa-Wagner et al., 2010 and Solaroglu et al., 2006), including the neonatal rat HI model (Fathali et al., 2010 and Zhao et al., 2007). Previously, Fathali et al. (2010) reported that G-CSF confers long-term neuroprotection by preventing brain atrophy and inducing somatic growth, as well as improving sensorimotor and neurocognitive functions, including limb placing, reflexes, short-term memory, muscle strength, exploratory behavior, and motor coordination in a neonatal HI rodent model.

Many pathways are activated by stimulating the G-CSF (G-CSFR), such as the Janus kinase (JAK)/signal transducer and activator of transcription (STAT) pathway, the Ras/mitogen activated protein kinase (MAPK) pathway, and the phosphoinositide 3-kinase (PI3K)/protein kinase B (Akt) pathway (Pap and Cooper, 1998 and Solaroglu et al., 2007). Of interest to this study, the PI3K/Akt pathway has been implicated in

stabilization of the blood-brain barrier (BBB) through decreased expression of VCAM-1 (Tsoyi et al., 2010), ICAM-1 (Radisavljevic et al. 2000), and b-catenin (Krafft et al., 2013), and increased expressions of Claudins 3 and 5 (Krafft et al., 2013).

Glycogen synthase kinase (GSK-3 $\beta$ ), which participates in a myriad of pathways throughout the central nervous system, can be either neuroprotective or neurodegenerative depending on the site of phosphorylation; GSK-3 $\beta$  activity is increased by phosphorylation of tyrosine-216 and decreased by serine-9 phosphorylation (Bhat et al., 2000, Grimes and Joep, 2001 and Valerio et al., 2011). While there is substantial evidence that GSK-3 $\beta$  inhibition (tyrosine-216 dephosphorylation) reduces neuronal apoptosis (Beurel and Joep, 2006, Krafft et al., 2012, Linseman et al., 2004 and Song et al., 2010), experimental evidence of GSK-3 $\beta$ 's effects on BBB stabilization and attenuation of inflammation are limited.

A number of studies have linked GSK-3 $\beta$  with the PI3K/Akt pathway, showing that phosphorylated Akt inactivates GSK-3 $\beta$  via tyrosine-216 dephosphorylation, decreasing neuronal apoptosis (Krafft et al., 2012 and Krafft et al., 2013). In this study, we investigate the role of G-CSF on the attenuation of inflammation and BBB disruption in a neonatal HI model using P10 rats. Specifically, we hypothesize that G-CSF will reduce pro-inflammatory markers, decrease adherens junction proteins, and increase tight junction proteins via inactivation of GSK-3 $\beta$  through the G-CSFR/PI3K/Akt pathway.

## **Materials and Methods**

### ***Neonatal Hypoxia-Ischemia Injury Model***

All of the experiments conducted in this study were approved by the Institutional

Animal Care and Use Committee of Loma Linda University. A total of 150 post-natal day 10 (P10) Sprague–Dawley rat pups (male and female), weighing 14–20 g, were used in this study. To prepare for the neonatal hypoxia-ischemia model, all animals were anesthetized with 3% isoflurane delivered with medical gas. After induction, confirmed by the loss of a paw pinch reflex and monitored throughout the procedure, surgery began.

Unilateral HI surgery techniques were employed as previously described with P10 rat pups (Burchell et al., 2013 and Fathali et al., 2010). P10 pups were underwent a unilateral right common carotid ligation. After surgery, animals recovered for 1 h and were then placed into a hypoxia chamber with 8% O<sub>2</sub> and 92% N<sub>2</sub>, which was submerged in a water bath maintained at 37 °C for 2.5 h. The flow rate of the gas was 93.82 mL/min for the first 1.25 h and 77.30 mL/min for the final 1.25 h. After hypoxia, all animals were returned to their dam.

The animal groups consisted of the following: (1) Sham, (2) HI, (3) HI + GSK-3 $\beta$  siRNA, (4) HI + Control siRNA, (5) HI + G-CSF, (6) HI + G-CSF + Control siRNA, (7) HI + G-CSF + G-CSFR siRNA, (8) HI + G-CSF + DMSO (Dimethyl sulfoxide) (vehicle for Wortmannin), and (9) HI + G-CSF + Wort (Wortmannin). Sham animals underwent the HI surgical procedures (i.e. exposure of the common carotid artery) without artery ligation and without exposure to hypoxic conditions.

### ***Experimental Conditions and Pharmacological Interventions***

The role of the G-CSFR/PI3K/Akt/GSK-3 $\beta$  signaling pathway for preventing BBB disruption and neuroinflammation after HI was examined using the following antagonists: G-CSFR siRNA, Control siRNA, Wortmannin, and GSK-3 $\beta$  siRNA.

After exposure to the hypoxia chamber, 134 injured animals were treated with G-CSF 50 µg/kg (Amgen Inc., Thousand Oaks, CA, USA) in PBS administered subcutaneously 1 h after completion of the HI procedure (1 h after pups are removed from the hypoxia chamber) (Doycheva et al., 2013). To understand the effects of various antagonists with G-CSF treatment, the following groups were studied: HI + G-CSF (no antagonist), HI + G-CSF + Control siRNA, HI + G-CSF + G-CSFR siRNA, HI + G-CSF + DMSO, and HI + G-CSF + Wortmannin.

For the HI + GSK-3β siRNA group, two different GSK-3β siRNAs (21500 R12-1717, R12-1719, Cell Signaling Technology, Inc. Danvers, MA, USA), with a total volume of 4 µL (2 µL of each) were stereotactically injected with a 10 µL syringe (Hamilton, Nevada, USA), 1.5 mm posterior and 1.5 mm lateral to the bregma and 1.7 mm deep from the skull in the ventricle of the contralateral hemisphere 48 h prior to HI. The HI surgery followed the procedure outlined above. The HI + Control siRNA group followed the same method except Control siRNA (4 µL, Santa Cruz Biotechnology, Inc., CA, USA) was also given.

For the HI + G-CSF + G-CSFR group, 2 µL of 0.4 nM G-CSFR siRNA (G-CSFR siRNA, Santa Cruz Biotechnology Inc. CA, USA, resuspended in RNA-free water) was administered 1.5 mm posterior and 1.5 mm lateral to the bregma, and 1.7 mm deep from the skull in the contralateral hemisphere 48 h prior to HI. HI surgery and G-CSF treatment were described above. The HI + G-CSF + Control siRNA group followed the same procedure except Control siRNA (2 µL, Santa Cruz Biotechnology, Inc., CA, USA) was used instead of the G-CSFR siRNA.

Wortmannin (86 ng/pup diluted in 2% DMSO, Cell Signaling Technology, Inc. Danvers, MA, USA) was administered 2.0 mm posterior and 2.0 mm lateral to the bregma, and 2.0 mm deep to the skull surface at the contralateral hemisphere 1 h prior to HI surgery. The same volume of 2% DMSO (diluted in saline, Sigma Aldrich, St. Louis, MO, USA) was administered as control.

***Time Course of  $\beta$ -catenin Phosphorylation,  $\beta$ -catenin, IKK $\beta$ , and NF- $\kappa$ B Expression after HI***

A total of 30 animals were sacrificed at 0, 12, 24, 48, or 72 h (n = 6 per time point) after HI surgery. Western blot was performed in order to investigate any changes in p- $\beta$ -catenin,  $\beta$ -catenin, IKK $\beta$ , and NF- $\kappa$ B following HI. The ipsilateral hemispheres were then isolated and analyzed for protein expressions.

***Expression of  $\beta$ -catenin Phosphorylation,  $\beta$ -catenin, IKK $\beta$ , and NF- $\kappa$ B 48 h following HI after GSK-3 $\beta$  siRNA***

Twelve animals were divided between the HI + Control siRNA and HI + GSK-3 $\beta$  siRNA groups (n = 6 in each group) in order to measure the expression of p- $\beta$ -catenin,  $\beta$ -catenin, IKK $\beta$ , and NF- $\kappa$ B after the administration of GSK-3 $\beta$  siRNA following HI. The ipsilateral hemispheres were isolated and analyzed by Western blot. The Sham and HI animals were shared with the 48 h time course expression groups.

### ***Evans' Blue Dye Extravasation***

Four percent Evans blue (EB) solution in 0.1 M PBS was injected subcutaneously (0.04 mL/15 g body weight) (Fernandez-Lopez et al., 2012) 26–28 h after HI and left to circulate for 20–22 h. The pups were then anesthetized and transcardially perfused with 50 mL of 0.1 M PBS, the brains removed and cut into 2 mm slices with a rat brain matrix 48 h after HI, then separated into the contralateral and ipsilateral hemispheres. The amount of EB in the ipsilateral hemisphere (weighed before homogenized) was measured with a spectrophotometer (Genesys 10S UV-Vis, Thermo Fisher Scientific Inc. Waltham, MA, USA) to evaluate blood–brain barrier (BBB) permeability. The Evans blue extravasation was quantified as  $\mu\text{g}$  Evans blue/g tissue using a standard curve. Evans blue data for all groups was normalized to the value of the Sham group.

### ***Immunohistochemistry***

Anesthetized pups were transcardially perfused with 0.1 M PBS followed by 4% formaldehyde solution (PFA) 48 h after HI. The brains were then removed, postfixed (4% PFA, 4 °C, 24 h), and then transferred into a 30% sucrose solution for 2 days. The cryoprotected brains were sectioned at a thickness of 10  $\mu\text{m}$  with a cryostat (LM3050S, Leica Microsystems Inc, IL, USA) for double fluorescence staining. The sections were then washed three times with 0.1 M PBS and were incubated with blocking solution (10% normal goat serum in 0.1 M PBS) for 2 h at room temperature following 0.1% Triton X-100 (37 °C, 30 min). Primary antibodies anti-GFAP (1:200, Millipore, Billerica, MA, USA), anti-vWF (1:200, Millipore, Billerica, MA, USA), anti-G-CSFR (1:100, Santa Cruz Biotechnology, Inc., CA, USA), anti-p-GSK-3 $\beta$  (Tyr216, 1:200,

abcam, Cambridge, MA, USA), anti- $\beta$ -catenin (1:200, abcam, Cambridge, MA, USA), anti-MPO (1:100, Santa Cruz Biotechnology, Inc., CA, USA), and DAPI (Vector Laboratories Inc. Burlingame, CA, USA) were applied (4 °C, overnight).

The sections were then washed with 0.1 M PBS and incubated for 2 h with secondary antibodies (1:200, anti-mouse IgG labeled with Alexa Fluor-488, anti-rabbit IgG labeled with Alexa Fluor-568, Jackson ImmunoResearch Laboratories, Inc. West Grove, PA, USA) at room temperature. The stained slices were observed with an OLYMPUS BX51 microscope.

### ***Western Blot***

Western blot was performed as described previously (Chen et al., 2008). Animals were euthanized at the various time points after HI. After intracardiac perfusion with cold PBS (pH 7.4) solution, the brains were removed and separation into ipsilateral and contralateral cerebrums. Samples were frozen with liquid nitrogen and stored at – 80 °C until analysis occurred. The whole-cell lysates were obtained by homogenizing with RIPA lysis buffer (sc-24948, Santa Cruz Biotechnology, Inc., TX, USA) and were centrifuged at 14,000 g at 4 °C for 30 min. The supernatant was used as a whole-cell protein extract, and the protein concentration was measured by using a detergent compatible assay (Bio-Rad, Dc protein assay). Equal amounts of protein (50  $\mu$ g) were loaded to a 10% SDS–PAGE gel. After being electrophoresed and transferred to a nitrocellulose membrane, the membrane was blocked with 5% non-fat blocking grade milk (Bio-Rad, Hercules, CA, USA) and incubated with the primary antibody overnight at 4 °C. The primary antibodies used were anti-actin (47 kDa, 1:4000, Santa Cruz



Biotechnology, Inc., CA, USA), anti-G-CSFR (90 kDa, 1:5000, Santa Cruz Biotechnology, Inc., CA, USA), anti-p-GSK-3 $\beta$  (Tyr216) (49 kDa), anti-GSK-3 $\beta$  (47 kDa), anti- $\beta$ -catenin (94 kDa), anti-p- $\beta$ -catenin (94 kDa, 1:10000, abcam, Cambridge, MA, USA), anti-MPO (150 kDa), anti-Claudin-3 (23 kDa), anti-p120-catenin (94 kDa), anti-Claudin-5 (23 kDa), anti-IL-1 $\beta$  (17.5 kDa), anti-IL-10 (18 kDa), anti-IL-12 (75 kDa), anti-IKK $\beta$  (87 kDa), anti-NF- $\kappa$ B (64 kDa) (1:4000, Santa Cruz Biotechnology, Inc., CA, USA), and anti-polyclonal antitumor necrosis factor- $\alpha$  (anti-TNF- $\alpha$ ) (52 kDa, 1:10000, Millipore, Billerica, MA, USA).

The nitrocellulose membranes were incubated with secondary antibodies (Santa Cruz Biotechnology, Inc., CA, USA) for 2 h at room temperature. Immunoblots were then probed via ECL Plus chemiluminescence reagent kit (Fisher Scientific International, Inc. Pittsburgh, PA, USA) and analyzed using Image J (4.0, Media Cybernetics, Silver Springs, MD). Western blot data were presented as the ratio of the target protein's pixel intensities to the pixel intensities of  $\beta$ -actin. For  $\beta$ -catenin, the pixels of p- $\beta$ -catenin were divided by the pixels of  $\beta$ -catenin (to obtain the ratio of  $\beta$ -catenin, which was phosphorylated to that of the total available  $\beta$ -catenin). The p- $\beta$ -catenin/ $\beta$ -catenin ratio was then normalized to the pixels of  $\beta$ -actin. All target protein ratios were then normalized to the Sham group's ratio of the pixel intensities.

### *Statistics*

All the data were expressed as mean  $\pm$  SEM. Statistical differences between groups for each outcome measured were analyzed using one-way ANOVA followed by Tukey post hoc. A  $p < 0.05$  was considered to be statistically significant.

## Result

### *Time Course Expression of p- $\beta$ -catenin/ $\beta$ -catenin, IKK $\beta$ and NF- $\kappa$ B after HI*

The ratio of p- $\beta$ -catenin/ $\beta$ -catenin and the expression of IKK $\beta$  and NF- $\kappa$ B in response to HI were examined at four times post-ictus (Fig. 1). Representative western blots are shown for each protein (Fig. 1D). The ratio of p- $\beta$ -catenin to  $\beta$ -catenin to  $\beta$ -actin (p- $\beta$ -catenin/  $\beta$ -catenin/ $\beta$ -actin) expression was low in Sham but, after HI, it gradually increased over 12 h ( $p < 0.05$  vs. Sham) to 24 h ( $p < 0.05$  vs. Sham), peaking significantly at 48 h ( $p < 0.05$  vs. Sham).

By 72 h, the p- $\beta$ -catenin/ $\beta$ -catenin/ $\beta$ -actin ratio had significantly decreased compared to the 48 h time point ( $p < 0.05$  vs. Sham, 12 h, 24h, and 48 h) (Fig. 1A). IKK $\beta$  and NF- $\kappa$ B showed similar expression patterns, in which both proteins' levels had low expression in the Sham group which significantly increased at 12 h ( $p < 0.05$  vs. Sham for IKK $\beta$ / $\beta$ -actin,  $p < 0.05$  vs. Sham for NF- $\kappa$ B/ $\beta$ -actin) reaching a maximum at 48 h ( $p < 0.05$  vs. Sham for IKK $\beta$ / $\beta$ -actin,  $p < 0.05$  vs. Sham for NF- $\kappa$ B/ $\beta$ -actin). Both IKK $\beta$ / $\beta$ -actin and NF- $\kappa$ B/ $\beta$ -actin began decreasing by 72 h ( $p < 0.05$  vs. Sham, 12 h, 24 h, and 48 h groups for IKK $\beta$ / $\beta$ -actin,  $p < 0.05$  vs. Sham, 12 h, 24 h, and 48 h groups for NF- $\kappa$ B/ $\beta$ -actin) (Fig. 1B, C).

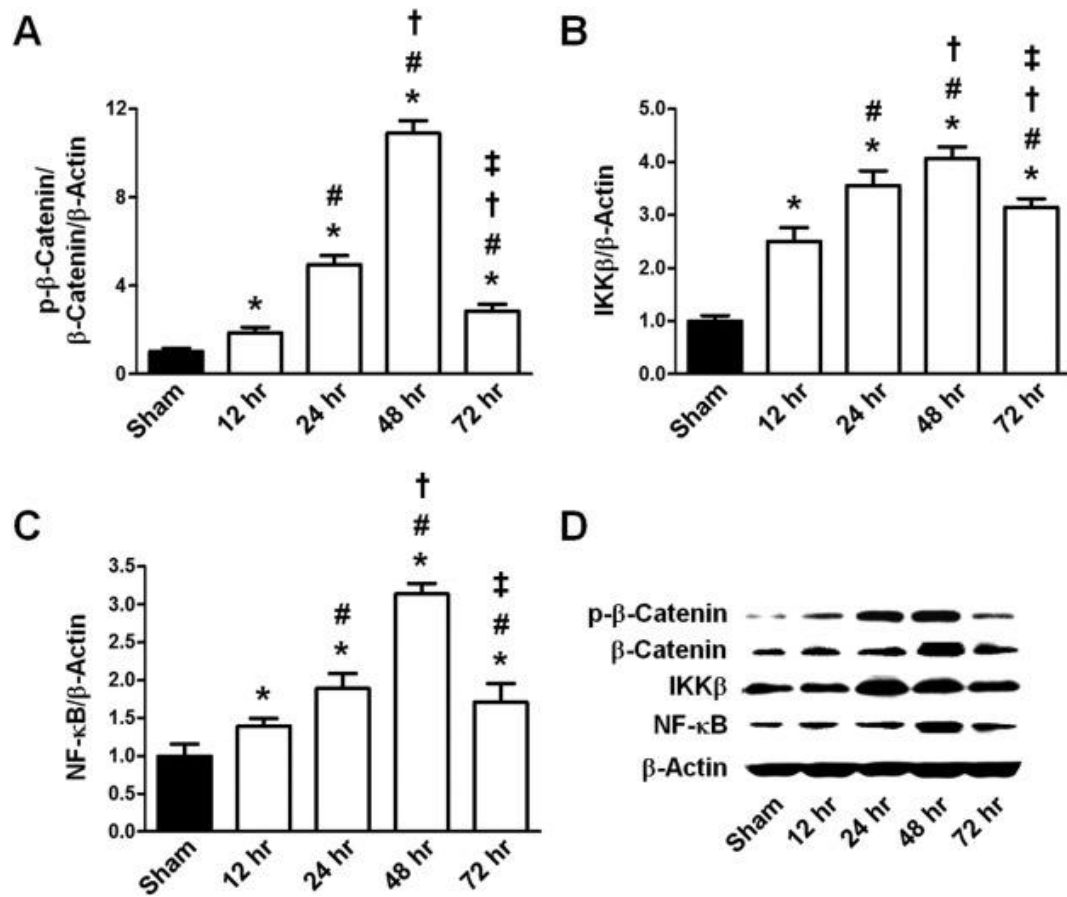


Fig. 14. Expression ratios of p-β-catenin/β-actin, β-catenin/β-actin, IKKβ/β-actin, and NF-κB/β-actin 12, 24, 48, and 72 h following HI. (A) Transient response of the p-β-catenin/β-actin ratio following HI (n = 6/group). (B) Transient response of IKKβ/β-actin ratio following HI (n = 6/group). (C) Transient response of NF-κB/β-actin ratio following HI (n = 6/group). (D) Representative Western blot of p-β-catenin, β-catenin, IKKβ, NF-κB, and β-actin for Sham and HI (12, 24, 48, and 72 h post-HI) animals. All graphs: \*p < 0.05 vs. Sham group, #p < 0.05 vs. 12 h post-HI group, †p < 0.05 vs. 24 h post-HI group, ‡p < 0.05 vs. 48 h post-HI group.

***Expression of p- $\beta$ -catenin/ $\beta$ -catenin, IKK $\beta$ , and NF- $\kappa$ B after GSK-3 $\beta$  siRNA Pre-treatment 48 h Post-HI***

The effects of GSK-3 $\beta$  siRNA on the expression levels of the p- $\beta$ -catenin/ $\beta$ -catenin ratio, IKK $\beta$ , and NF- $\kappa$ B were measured (Fig. 2). The levels of p- $\beta$ -catenin/ $\beta$ -catenin/ $\beta$ -actin, IKK $\beta$ / $\beta$ -actin, and NF- $\kappa$ B/ $\beta$ -actin expression in the HI groups were significantly higher when compared to the Sham levels ( $p < 0.05$  vs. Sham for p- $\beta$ -catenin/ $\beta$ -catenin/ $\beta$ -actin, IKK $\beta$ / $\beta$ -actin, and NF- $\kappa$ B/ $\beta$ -actin). After HI, GSK-3 $\beta$  knockdown by siRNA significantly reduced the expression of these proteins compared to HI ( $p < 0.05$  vs. HI for p- $\beta$ -catenin/ $\beta$ -catenin/ $\beta$ -actin, IKK $\beta$ / $\beta$ -actin, and NF- $\kappa$ B/ $\beta$ -actin), while control siRNA did not alter any of these proteins' expressions compared to HI ( $p > 0.05$  vs. HI for p- $\beta$ -catenin/ $\beta$ -catenin/ $\beta$ -actin, IKK $\beta$ / $\beta$ -actin, and NF- $\kappa$ B/ $\beta$ -actin).

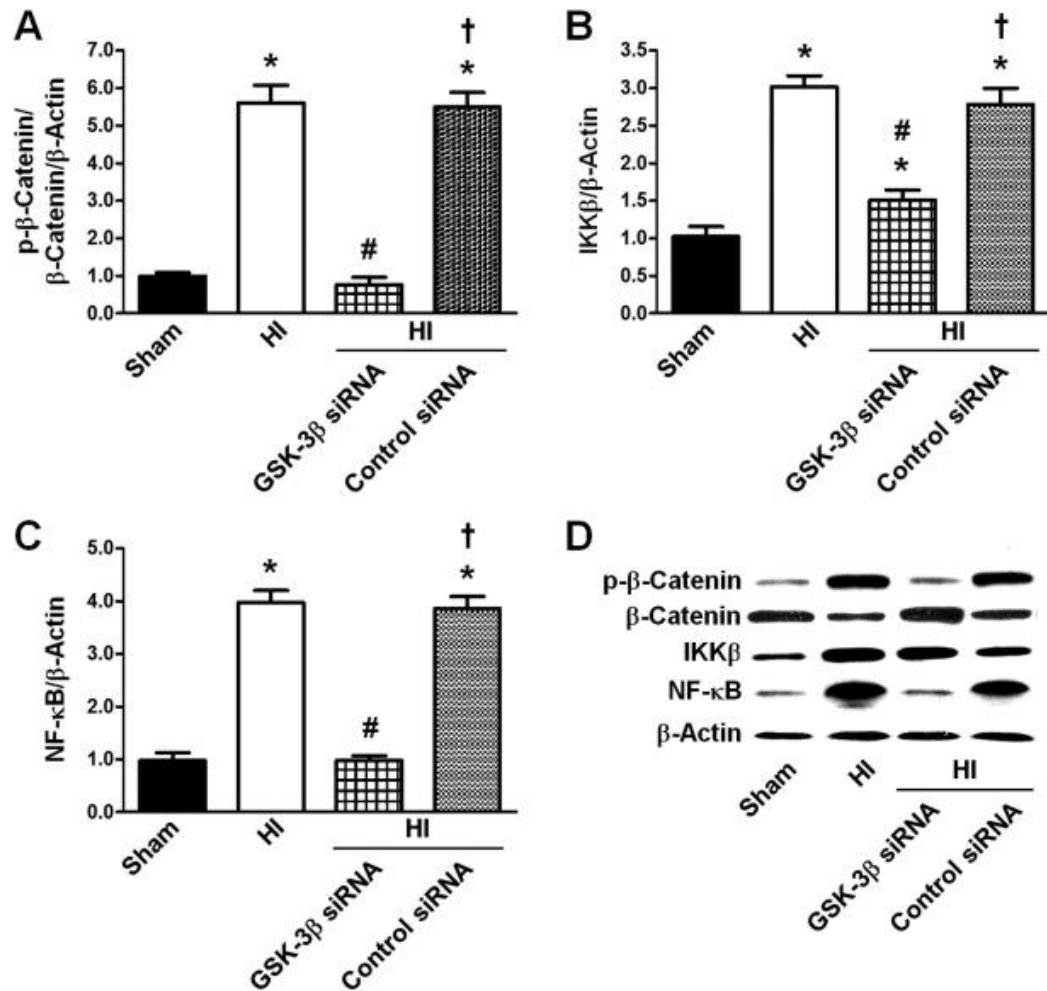


Fig. 15. Effect of GSK-3 $\beta$  siRNA on the p- $\beta$ -catenin/ $\beta$ -catenin/ $\beta$ -actin and IKK $\beta$ / $\beta$ -actin and NF- $\kappa$ B/ $\beta$ -actin expression ratios 48 h after HI. (A) Effect of GSK-3 $\beta$  siRNA on the p- $\beta$ -catenin/ $\beta$ -catenin/ $\beta$ -actin ratio (n = 6/group). (B) Effect of GSK-3 $\beta$  siRNA on IKK $\beta$ / $\beta$ -actin ratio (n = 6/group). (C) Effect of GSK-3 $\beta$  siRNA on the NF- $\kappa$ B/ $\beta$ -actin ratio (n = 6/group). (D) Representative western blot of p- $\beta$ -catenin,  $\beta$ -catenin, IKK $\beta$ , NF- $\kappa$ B, and  $\beta$ -actin for Sham, HI, HI + GSK-3 $\beta$  siRNA, and HI + Control siRNA animals. All graphs: \*p < 0.05 vs. Sham group, #p < 0.05 vs. HI only group, †p < 0.05 vs. HI + GSK-3 $\beta$  siRNA group.

### ***G-CSF Reduced Evans Blue Extravasation 48 h after HI***

The amount of ipsilateral hemisphere Evans blue extravasation was significantly higher in the HI group compared to Sham ( $p < 0.05$ ) (Fig. 3). Both G-CSF treatment and GSK-3 $\beta$  knockdown by siRNA significantly reduced the extravasation of Evans blue compared to HI only ( $p < 0.05$  vs. HI for HI + G-CSF,  $p < 0.05$  vs. HI for HI + GSK-3 $\beta$  siRNA). The neuroprotective effects of G-CSF after HI were unaffected by DMSO or control siRNA ( $p < 0.05$  vs. HI for DMSO,  $p < 0.05$  vs. HI for control siRNA,  $p > 0.05$  vs. HI + G-CSF for DMSO,  $p > 0.05$  vs. HI + G-CSF for control siRNA). The inhibition of Akt by Wortmannin and G-CSFR knockdown by siRNA completely prevented the neuroprotective effects of G-CSF after HI ( $p < 0.05$  vs. Sham, HI + G-CSF, and HI + GSK-3 $\beta$  siRNA for Wortmannin,  $p < 0.05$  vs. Sham, HI + G-CSF, and HI + GSK-3 $\beta$  siRNA for G-CSFR siRNA).

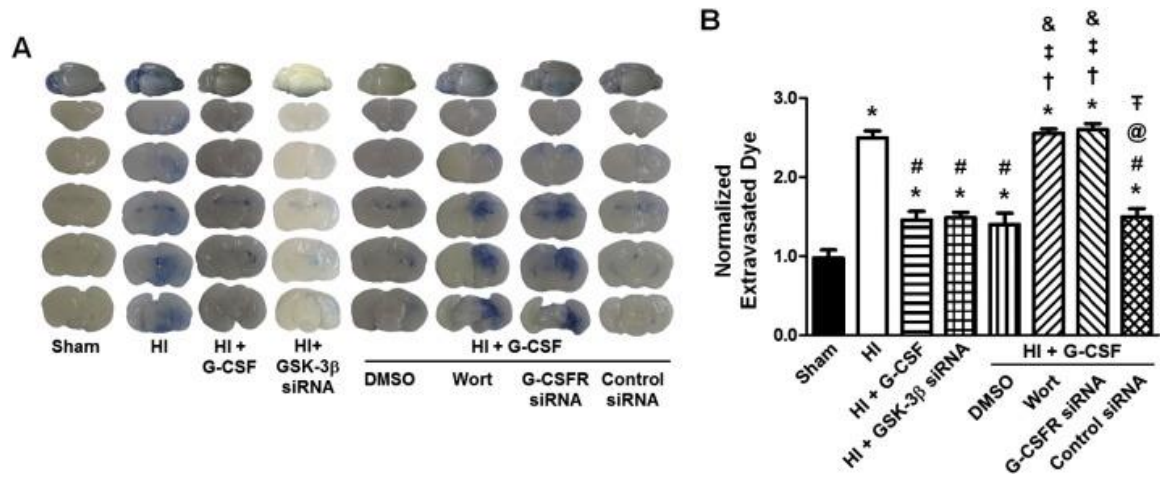


Fig. 16. Evans blue extravasation 48 h after HI. Brain slices with extravasated dye demonstrate the effect of G-CSF (administered 1 h after removal from the hypoxia chamber) and G-CSF/GSK-3 $\beta$  pathway inhibitors on BBB permeability. The extravasated dye from the brain slices is quantitatively evaluated ( $\mu$ g of Evan's Blue dye per g of brain tissue, normalized to Sham) ( $n = 6$ /group). Graph: \* $p < 0.05$  vs. Sham group, # $p < 0.05$  vs. HI only group, † $p < 0.05$  vs. HI + G-CSF group, ‡ $p < 0.05$  vs. HI + GSK-3 $\beta$  siRNA group, & $p < 0.05$  vs. HI + G-CSF + DMSO group, @ $p < 0.05$  vs. HI + G-CSF + Wortmannin, ‡ $p < 0.05$  vs. HI + G-CSFR siRNA group.

***Immunohistochemistry Showed Co-localization of G-CSFR with Endothelial cells 48 h  
after HI***

Immunohistochemical analysis showed that G-CSFR localizes on endothelial cells (vWF) but not astrocytes (GFAP) and p-GSK-3 $\beta$  also localizes within endothelial cells. These results show that both the G-CSFR and p-GSK3 $\beta$  are localized in endothelial cells (Fig. 4).



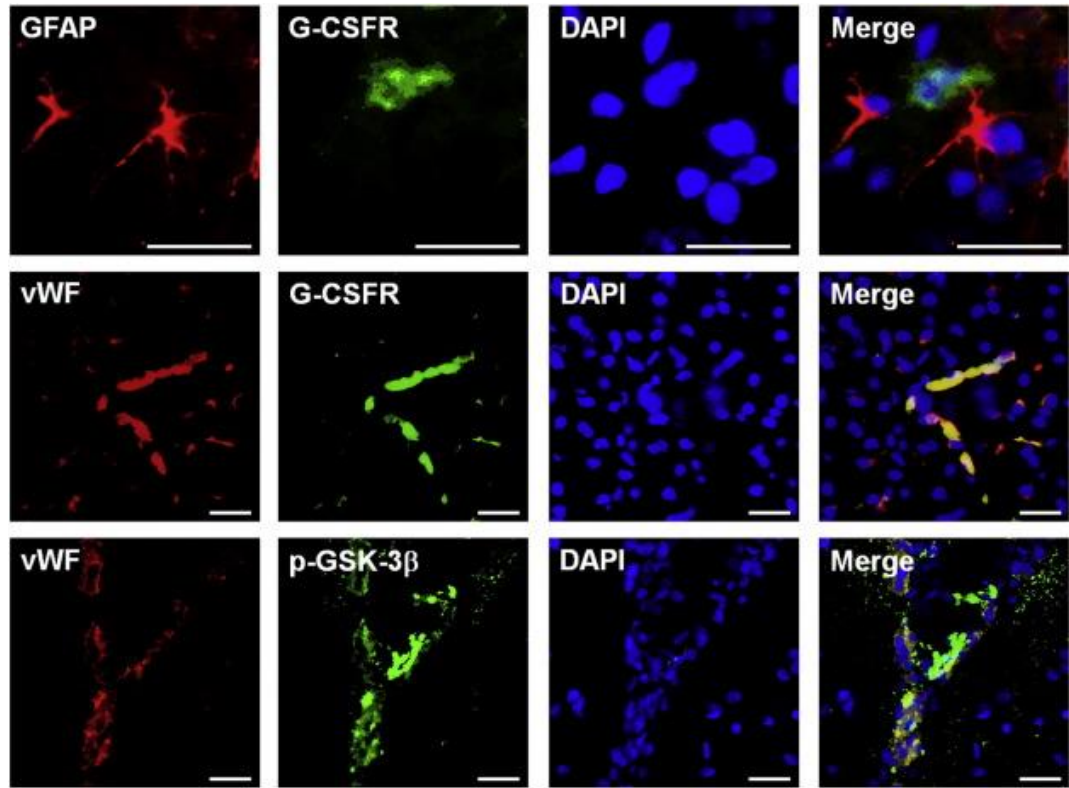


Fig. 17. Expression and localization of G-CSFR 48 h following HI. Top row: representative immunohistochemistry images showing G-CSFR does not localize on astrocytes (GFAP: red, G-CSFR: green, DAPI: blue). Middle row: representative immunohistochemistry images showing localization of G-CSFR on blood vessels (vWF: red, G-CSFR: green, DAPI: blue). Bottom row: representative immunohistochemistry images showing localization of p-GSK-3 $\beta$  on blood vessels (vWF: red, p-GSK-3 $\beta$ : green, DAPI: blue) (n = 3/group). Scale bars: 25  $\mu$ m.

### ***Expression of p- $\beta$ -catenin/ $\beta$ -catenin and p120-catenin 48 h after HI***

The ratio of p- $\beta$ -catenin/ $\beta$ -catenin/ $\beta$ -actin was significantly increased in the HI group compared to Sham ( $p < 0.05$  vs. Sham), which was decreased by G-CSF treatment ( $p < 0.05$  vs. HI). Wortmannin and G-CSFR siRNA both prevented the decreased expression by G-CSF treatment and also showed no changes in the ratio of p- $\beta$ -catenin/ $\beta$ -catenin/ $\beta$ -actin when compared to HI ( $p > 0.05$  vs. HI for Wortmannin,  $p > 0.05$  vs. HI for G-CSFR siRNA,  $p < 0.05$  vs. HI + G-CSF for Wortmannin,  $p < 0.05$  vs. HI + G-CSF for G-CSFR siRNA) (Fig. 5A, C).

p120-catenin/ $\beta$ -actin levels decreased after HI ( $p < 0.05$  vs. Sham) but were restored to Sham levels with G-CSF treatment ( $p > 0.05$  vs. Sham,  $p < 0.05$  vs. HI). Wortmannin and G-CSFR siRNA lowered p120-catenin/ $\beta$ -actin levels to those of the HI group ( $p < 0.05$  vs. Sham and HI + G-CSF for Wortmannin,  $p < 0.05$  vs. Sham and HI + G-CSF for G-CSFR siRNA,  $p > 0.05$  vs. HI for Wortmannin,  $p > 0.05$  vs. HI for G-CSFR siRNA) (Fig. 5B, C).

Representative immunohistochemical images showed decreased expression of  $\beta$ -catenin in the HI, HI + G-CSF + Wort, and HI + G-CSF + G-CSFR siRNA groups compared to Sham, while G-CSF treatment and GSK-3 $\beta$  knockdown by siRNA had similar expressions as Sham (Fig. 5E).

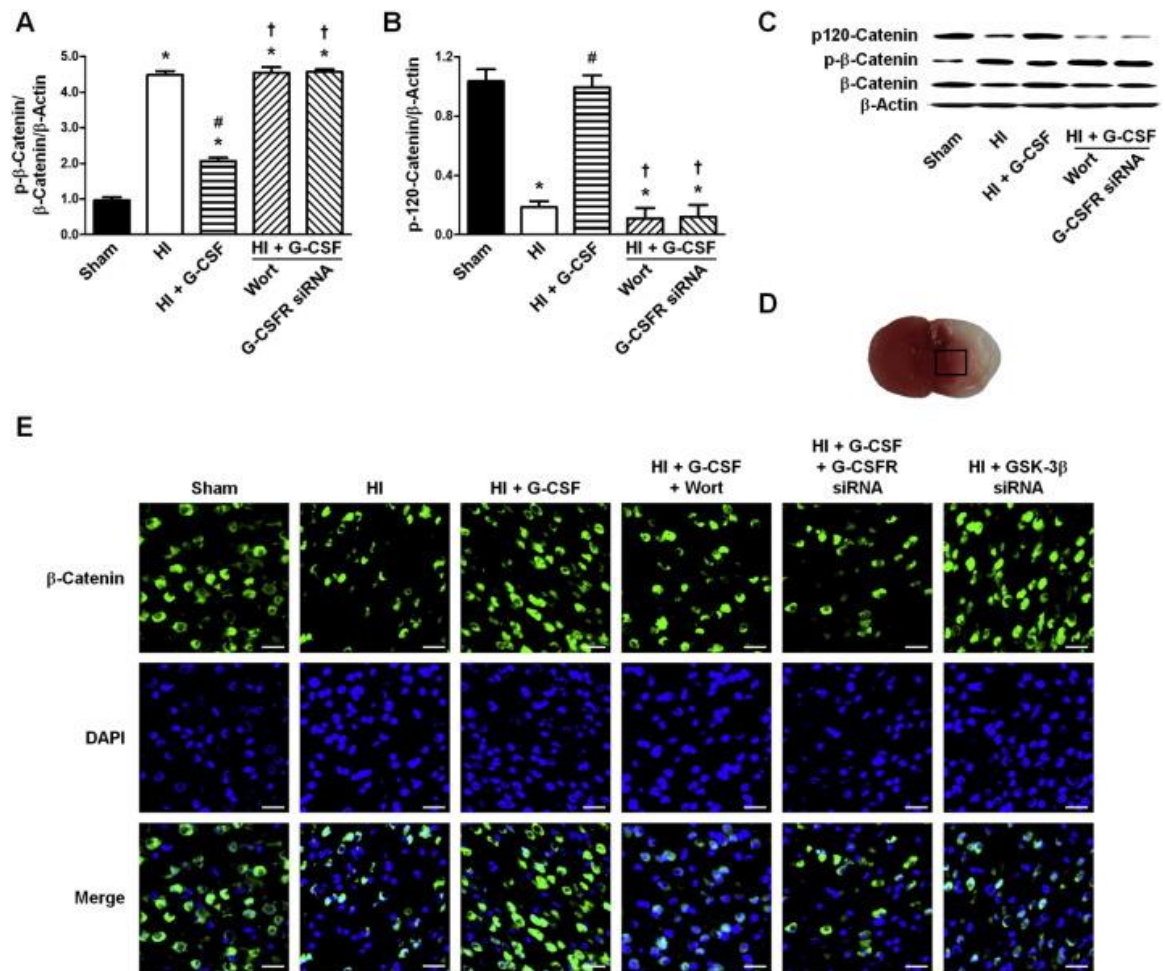


Fig. 18. Effect of G-CSFR siRNA on the p120-catenin/β-actin and p-β-catenin/β-catenin/β-actin expression ratios 48 h following HI. (A) Effect of G-CSFR siRNA on the p-β-catenin/β-catenin/β-actin ratio (n = 6/group). (B) Effect of G-CSFR siRNA on the expression ratio of p120-catenin/β-actin (n = 6/group). (C) Representative western blot of p-β-catenin, β-catenin, p120-catenin, and β-actin for Sham, HI only, HI + G-CSF, HI + G-CSF + Wortmannin, and HI + G-CSF + G-CSFR siRNA animals. (D) Representative brain slice (stained with TTC, red color indicates non-ischemic tissue, white color indicates ischemic tissue) and area (box) used for immunohistochemistry imaging. (E) Representative immunohistochemistry images for β-catenin expression following HI. Scale bars: 25 μm. Graph: \*p < 0.05 vs. Sham group, #p < 0.05 vs. HI only group, †p < 0.05 vs. HI + G-CSF group.

### ***Claudin-3 and Claudin-5 Expressions 48 h after HI***

Both Claudin-3/ $\beta$ -actin and Claudin-5/ $\beta$ -actin expression levels were decreased by HI compared to Sham ( $p < 0.05$  vs. Sham for Claudin-3/ $\beta$ -actin,  $p < 0.05$  vs. Sham for Claudin-5/ $\beta$ -actin), while G-CSF treatment restored both claudins' levels back to normal ( $p > 0.05$  vs. Sham and  $p < 0.05$  vs. HI for Claudin-3/ $\beta$ -actin,  $p > 0.05$  vs. Sham and  $p < 0.05$  vs. HI for Claudin-5/ $\beta$ -actin). Wortmannin and G-CSF siRNA similar expression levels of Claudin-3/ $\beta$ -actin as HI group, preventing the effect of G-CSF ( $p < 0.05$  vs. Sham and HI + G-CSF for Wortmannin,  $p < 0.05$  vs. Sham and HI + G-CSF for G-CSFR siRNA,  $p > 0.05$  vs. HI for Wortmannin,  $p > 0.05$  vs. HI for G-CSFR siRNA). Claudin-5/ $\beta$ -actin expression was lower for both Wortmannin and G-CSFR knockdown by siRNA than the Sham, HI, and HI + G-CSF groups ( $p < 0.05$  vs. Sham, HI, and HI + G-CSF for Wortmannin,  $p < 0.05$  vs. Sham, HI, and HI + G-CSF for G-CSFR siRNA) (Fig. 6).

### ***Expression of MPO, VCAM-1, and ICAM-1 48 h after HI***

The level of MPO/ $\beta$ -actin was significantly increased in the HI group compared to Sham ( $p < 0.05$  vs. Sham). G-CSF treatment restored the MPO/ $\beta$ -actin level back to Sham levels ( $p > 0.05$  vs. Sham,  $p < 0.05$  vs. HI). The inhibition of Akt by Wortmannin and G-CSFR knockdown by siRNA both reversed G-CSF's neuroprotective effect by increasing MPO/ $\beta$ -actin levels back to that of HI ( $p < 0.05$  vs. Sham and HI + G-CSF for Wortmannin,  $p < 0.05$  vs. Sham and HI + G-CSF for G-CSFR siRNA,  $p > 0.05$  vs. HI for Wortmannin,  $p > 0.05$  vs. HI for G-CSFR siRNA) (Fig. 7A).

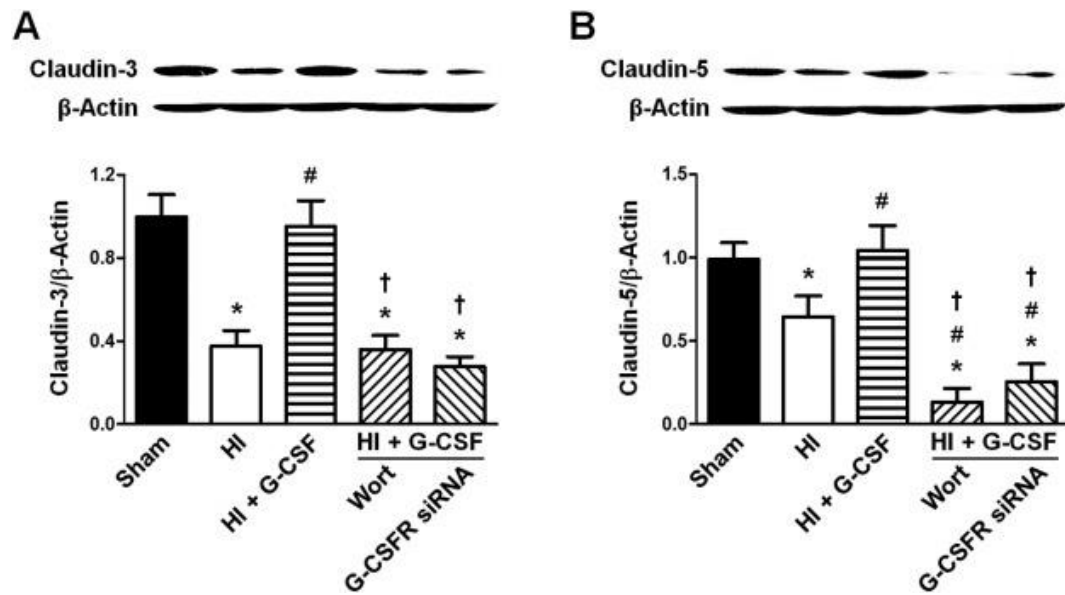


Fig. 19. Effect of G-CSFR siRNA on the ratios of Claudin-3/ $\beta$ -actin and Claudin-5/ $\beta$ -actin 48 h following HI. (A) Effect of G-CSFR siRNA on the ratio of Claudin-3/ $\beta$ -actin ( $n = 6/\text{group}$ ). (B) Effect of G-CSFR siRNA on the ratio of Claudin-5/ $\beta$ -actin ( $n = 6/\text{group}$ ). Both Graphs: \* $p < 0.05$  vs. Sham group, # $p < 0.05$  vs. HI only group, † $p < 0.05$  vs. HI + G-CSF group.

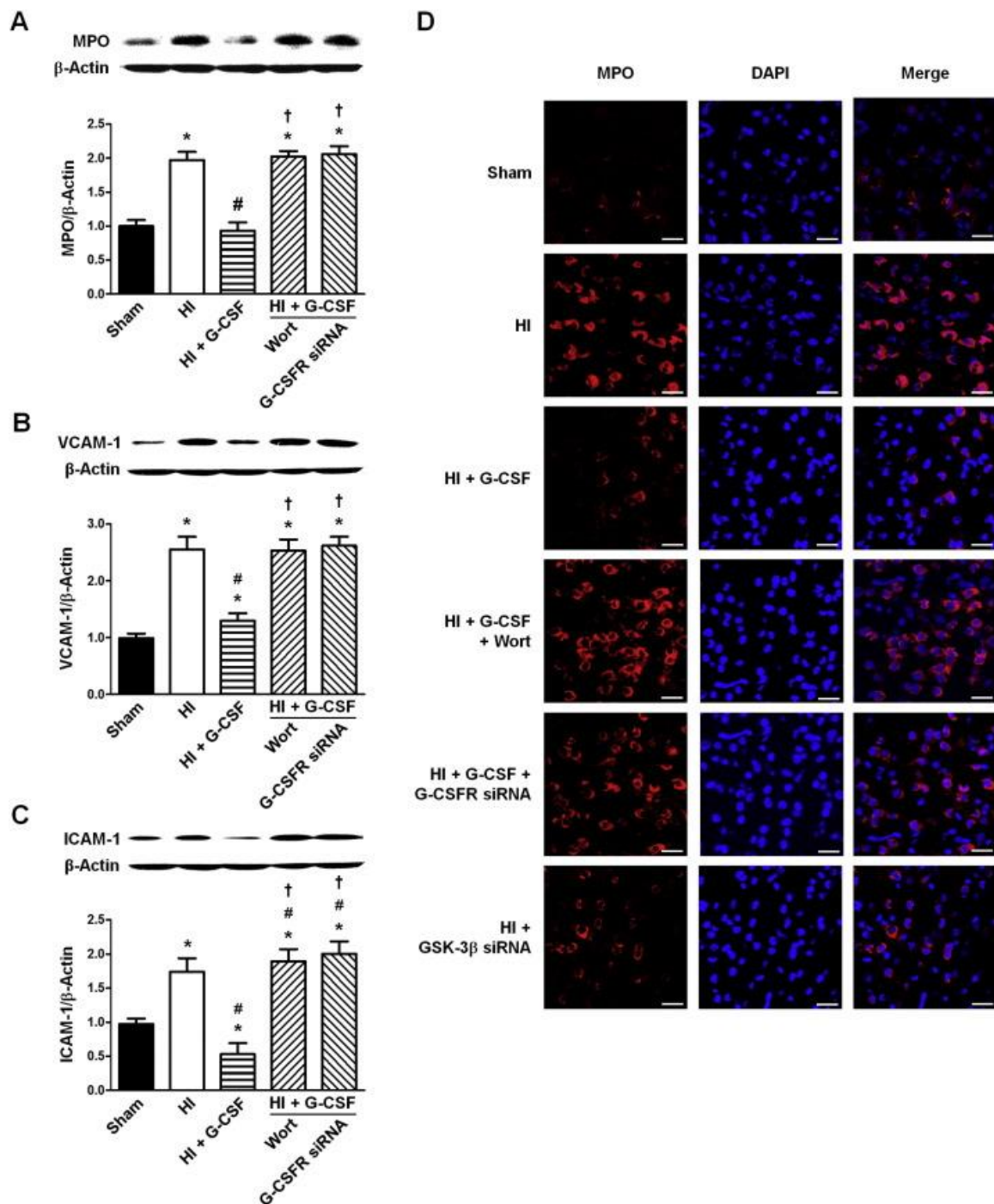


Fig. 20. Effect of G-CSFR siRNA on the ratios of VCAM-1/ $\beta$ -actin and ICAM-1/ $\beta$ -actin 48 h following HI. (A) Effect of G-CSFR siRNA on the ratio of MPO/ $\beta$ -actin ( $n = 6$ /group). (B) Effect of G-CSFR siRNA on the ratio of VCAM-1/ $\beta$ -actin ( $n = 6$ /group). (C) Effect of G-CSFR siRNA on the ratio of ICAM-1/ $\beta$ -actin ( $n = 6$ /group). (D) Representative immunohistochemistry images for MPO/ $\beta$ -actin ratio following HI. Scale bars: 25  $\mu$ m. The area used for immunohistochemistry imaging is the same as that in Fig. 5D. All graphs: \* $p < 0.05$  vs. Sham group, # $p < 0.05$  vs. HI only group, † $p < 0.05$  vs. HI + G-CSF group.

HI significantly increased both VCAM-1/ $\beta$ -actin and ICAM-1/ $\beta$ -actin adhesion molecule levels compared to Sham ( $p < 0.05$  vs. Sham for VCAM-1/ $\beta$ -actin,  $p < 0.05$  vs. Sham for ICAM-1/ $\beta$ -actin). Both adhesion molecules' expressions were decreased by G-CSF ( $p < 0.05$  vs. HI for VCAM-1/ $\beta$ -actin,  $p < 0.05$  vs. HI for ICAM-1/ $\beta$ -actin). Once again Wortmannin-induced Akt inhibition and G-CSFR knockdown reversed G-CSF's neuroprotective effect and the levels of VCAM-1/ $\beta$ -actin, and ICAM-1/ $\beta$ -actin remained unchanged compared to HI group ( $p < 0.05$  vs. Sham and HI + G-CSF for VCAM-1/ $\beta$ -actin,  $p < 0.05$  vs. Sham and HI + G-CSF for ICAM-1/ $\beta$ -actin,  $p > 0.05$  vs. HI for VCAM-1/ $\beta$ -actin,  $p > 0.05$  vs. HI for ICAM-1/ $\beta$ -actin) (Fig. 7B, C).

Immunohistochemical analysis was performed (brain area shown in Fig. 5D) on the above mentioned groups for MPO showed the same trend as that from the western blots. Furthermore, GSK-3 $\beta$  siRNA showed a decreased expression of MPO compared to HI group (Fig. 7D).

#### ***Expression of IKK $\beta$ , NF- $\kappa$ B, TNF- $\alpha$ , IL-1 $\beta$ , IL-10, and IL-12 48 h after HI***

The expression levels of IKK $\beta$ / $\beta$ -actin, NF- $\kappa$ B/ $\beta$ -actin, TNF- $\alpha$ / $\beta$ -actin, IL-1 $\beta$ / $\beta$ -actin, and IL-12/ $\beta$ -actin were significantly increased after HI compared to Sham ( $p < 0.05$  vs. Sham for all expressions) while G-CSF treatment reduced the effect ( $p < 0.05$  vs. HI for all expressions,  $p < 0.05$  vs. Sham for all expressions). Neither Wortmannin nor G-CSFR knockdown changed the expression levels of these targets compared to HI ( $p > 0.05$  vs. HI for all expressions for Wortmannin,  $p > 0.05$  vs. HI for all expression for G-CSFR siRNA,  $p < 0.05$  vs. Sham and G-CSF for all expressions for Wortmannin,  $p < 0.05$  vs. Sham and G-CSF for all expressions for G-CSFR siRNA) (Fig. 8A–D, F).

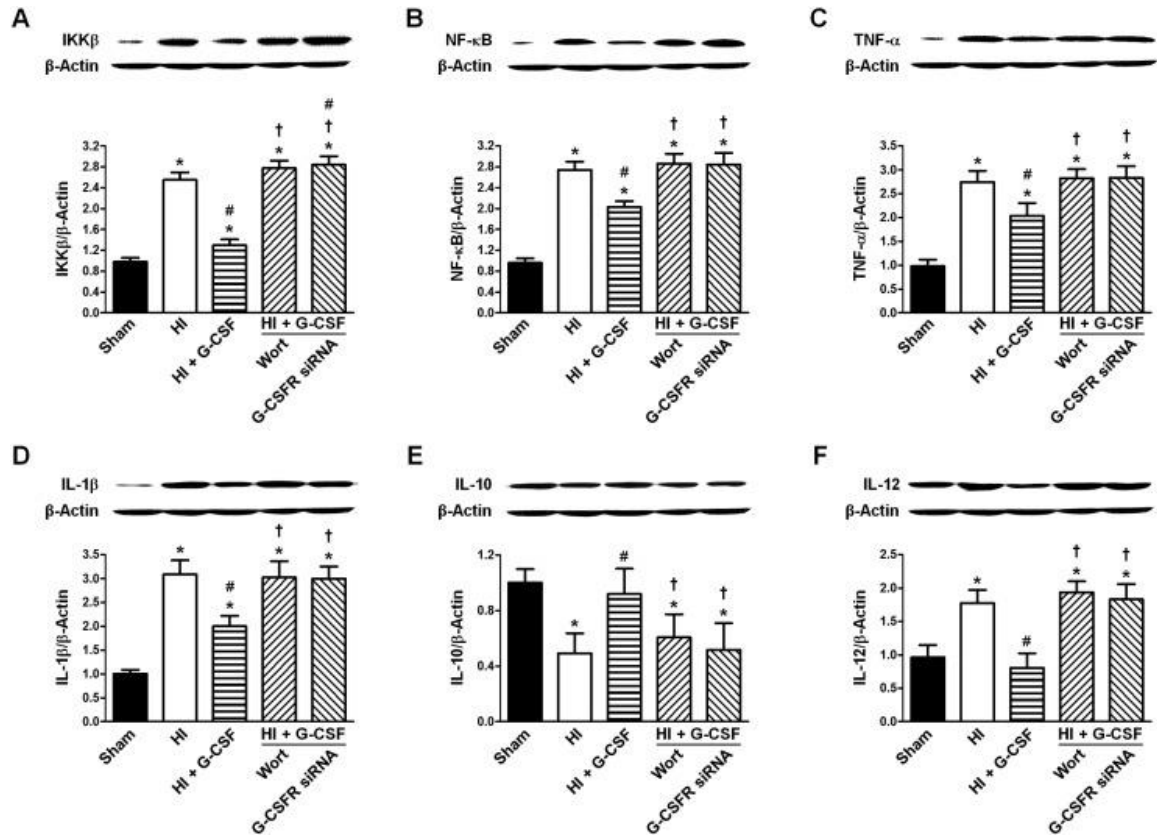


Fig. 21. Effect of G-CSFR siRNA on the ratios of IKKβ/β-actin, NF-κB/β-actin, TNF-α/β-actin, IL-1β/β-actin, IL-10/β-actin, and IL-12/β-actin 48 h following HI. (A) Effect of G-CSFR siRNA on the ratio of IKKβ/β-actin (n = 6/group). (B) Effect of G-CSFR siRNA on the ratio of NF-κB/β-actin (n = 6/group). (C) Effect of G-CSFR siRNA on the ratio of TNF-α/β-actin (n = 6/group). (D) Effect of G-CSFR siRNA on the ratio of IL-1β/β-actin (n = 6/group). (E) Effect of G-CSFR siRNA on the ratio of IL-10/β-actin (n = 6/group). (F) Effect of G-CSFR siRNA on the ratio of IL-12/β-actin (n = 6/group). All graphs: \*p < 0.05 vs. Sham group, #p < 0.05 vs. HI only group, †p < 0.05 vs. HI + G-CSF group.



Simultaneously, IL-10/ $\beta$ -actin was significantly decreased in the HI group ( $p < 0.05$  vs. Sham) and increased to Sham levels after G-CSF treatment ( $p > 0.05$  vs. Sham,  $p < 0.05$  vs. HI). Once again, Wortmannin and G-CSFR knockdown had similar expression levels of IL-10/ $\beta$ -actin as the HI group ( $p < 0.05$  vs. Sham for Wortmannin,  $p > 0.05$  vs. HI for Wortmannin,  $p < 0.05$  vs. Sham for G-CSFR siRNA,  $p > 0.05$  vs. HI for G-CSFR siRNA) (Fig. 8E).

## Discussion

In this study, G-CSF was found to stabilize the BBB and reduce a number of inflammatory markers. Various interventions of the proposed G-CSF/Akt/GSK-3 $\beta$  pathway were utilized, all of which prevented G-CSF's protective and anti-inflammatory effects.

G-CSF, a widely studied growth factor for stroke treatment, is neuroprotective in rodent models of cerebral ischemia (Schabitz et al., 2003, Shyu et al., 2004 and Yanqing et al., 2006) and neonatal hypoxia-ischemia (Doycheva et al., 2013). The anti-apoptotic effects of G-CSF are reported to reduce infarct volume and improve neurological outcomes after experimental cerebral ischemia (Gibson et al., 2005 and Schabitz et al., 2003) and neonatal hypoxia-ischemia (Doycheva et al., 2013). While G-CSF administration has been proven to reduce infarct volume, studies reporting the effects of G-CSF on brain edema and BBB after injury are limited and conflicting. In a rat model of traumatic brain injury, G-CSF was ineffective at reducing brain edema (Sakowitz et al., 2006). However, Gibson et al. (2005) found that G-CSF reduces cerebral edema after

middle cerebral artery occlusion in mice. Furthermore, the results by Gibson et al. (2005) suggest that G-CSF suppresses IL-1 $\beta$  mRNA expression.

Our current study examined a potential mechanism by which G-CSF treatment attenuates BBB disruption and neuroinflammation in a neonatal rat model of hypoxia-ischemia. More specifically, G-CSF elicited BBB stabilization by G-CSFR stimulation and activation of the PI3K/Akt pathway and resultant downstream inactivation of GSK-3 $\beta$  (Pap and Cooper, 1998 and Solaroglu et al., 2007). Our lab has recently demonstrated that phosphorylation of tyrosine-216 in GSK-3 $\beta$  plays a crucial role in regulating neuronal apoptosis through caspase-3 in a mouse model of intracerebral hemorrhage (Krafft et al., 2012). Additionally, activated Akt (p-Akt) has the ability to phosphorylate serine-9, which inactivates GSK-3 $\beta$  and reduces the amount of GSK-3 $\beta$  available for activation (through the tyrosine-216 form), ultimately decreasing apoptosis (Hummler et al., 2013 and Krafft et al., 2012). The inactivation of GSK-3 $\beta$ , specifically through serine-9 phosphorylation and tyrosine-216 dephosphorylation, increased  $\beta$ -catenin, an important factor in maintaining the BBB (Lin et al., 2009). Furthermore, GSK-3 $\beta$  inactivation may also decrease NF- $\kappa$ B expression, thereby reducing neuroinflammation (Krafft et al., 2013).

Findings in the present study indicate that G-CSF is capable of reducing BBB disruption through decreased  $\beta$ -catenin phosphorylation and p120-catenin phosphorylation via G-CSFR/PI3K/Akt/GSK-3 $\beta$  pathway. G-CSFR siRNA was associated with increased  $\beta$ -catenin activation and decreased activation of p120-catenin, while GSK-3 $\beta$  siRNA had the reverse effects. Evidence supporting enhanced BBB stabilization by G-CSF-induced G-CSFR/PI3K/Akt/GSK-3 $\beta$  pathway activation in

endothelial cells includes decreased adheren (VCAM-1 and ICAM-1) and increased tight junction (Claudin3 and 5) proteins. Together, our findings indicate that the activation of the G-CSFR/Akt/GSK-3 $\beta$  pathway by G-CSF plays a critical role in BBB stabilization.

We additionally examined the role G-CSF plays in modulating neuroinflammation by measuring expression levels of several cytokines; administering G-CSF attenuated pro-inflammatory cytokines (IKK $\beta$ , NF- $\kappa$ B, TNF- $\alpha$ , IL-1 $\beta$ , and IL-12) and enhanced an anti-inflammatory cytokine (IL-10). Our results agree with those by Gibson et al. (2005), indicating that G-CSF suppresses pro-inflammatory cytokines. The assessment of neutrophil infiltration via MPO staining shows decreased MPO expression following G-CSF treatment. The G-CSF-induced suppression of pro-inflammatory markers and the upregulation of anti-inflammatory markers are reversed by G-CSFR siRNA, further implicating the G-CSFR/Akt/GSK-3 $\beta$  pathway in preventing neuroinflammation after HI.

### ***Are Additional Studies on G-CSF Required?***

Recent experimental studies, including this one, indicate that G-CSF treatment remains a promising therapy due to its anti-apoptotic, anti-inflammatory, and BBB stabilizing effects. Although G-CSF has been largely successful in experimental models of ischemic brain injury, G-CSF failed to improve clinical outcome of adult ischemic stroke patients in the recent AX2000 clinical trial (Ringelstein et al., 2013). Possible explanations for its clinical failure include a reduced therapeutic effect from G-CSF due to rt-PA, an excessively long therapeutic window, and a limited number of centers involved in the trial (Ringelstein et al., 2013). First, the administration of rt-PA may

reduce G-CSF's beneficial effects by weakening the endothelium. Second, the length of time until treatment with G-CSF was 6.8 h post-ictus. By this time, a significant amount of irreversible damaged will have already occurred (An et al., 2014 and Lapchak, 2013), thus G-CSF's therapeutic benefit will be severely limited.

Additionally, a significant number of pathophysiological differences exist between adults and newborns, including differences in their responses to hypoxic ischemic injury and its treatments (Blomgren and Hagberg, 2013, Vexler et al., 2006a and Vexler et al., 2006b). Finally, G-CSF provides increased neuroprotection from ischemic brain injury in neonates (Charles et al., 2012) compared to adults (Solaroglu et al., 2009); G-CSF treatment (50 µg/kg) caused a 50% reduction in infarction volume following neonatal HI (Charles et al., 2012) compared to a 22% reduction following adult cerebral ischemia (Solaroglu et al., 2009).

There are three ongoing clinical trials evaluating G-CSF treatment for stroke: “Study to determine the effect of a drug called Neupogen on stroke recovery (GIST),” “Establishment of clinical basis for hematopoietic growth factors therapy in brain injury,” and “The variation of movement related cortical potential, cortio-cortical inhibition, and motor evoked potential in intracerebral implantation of antologous peripheral blood stem cells (CD34) in old ischemic stroke” (Liu et al., 2014b). However, all three of these trials examine G-CSF treatment in adult patients, and none have yet released results on G-CSF's therapeutic benefit.

In the current study, G-CSF plays a critical role in preventing BBB disruption and neuroinflammation via G-CSFR/PI3K/Akt pathway activation and downstream inactivation of GSK-3β. G-CSFR, previously reported to be expressed on neurons, is also

expressed on other cells of the neurovascular unit, supporting it as a promising therapeutic target (Leak et al., 2014). Combined with the limitations from the AX2000 clinical trial, this study warrants continued research on G-CSF treatment, possibly by using large animal models (Ramanantsoa et al., 2013 and Tajiri et al., 2013) following the guidelines for effective translational research (Chen et al., 2014 and Lapchak et al., 2013) as well as examining G-CSF's neuroprotective effects after ischemic brain injury in another clinical trial. Finally, future clinical trials should examine G-CSF's effects after hypoxic ischemia in newborns.

### **Conclusion**

Here we report attenuation of neuroinflammation and BBB disruption by G-CSF-induced G-CSFR/PI3K/Akt pathway activation and subsequent GSK-3 $\beta$  inactivation. G-CSFR was localized to endothelial cells but not astrocytes. Future studies should include determining whether G-CSF stabilizes the BBB, thereby reducing peripheral immune cell infiltration, or G-CSF reduces immune cell infiltration, thus maintaining BBB integrity. Additional studies should also include examining the role of G-CSF in reducing cerebral edema and the mechanism(s) by which G-CSF-mediated GSK-3 $\beta$  inactivation decreases VCAM and ICAM and increases the expression of claudins.

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**CHAPTER FIVE**  
**OSTEOPONTIN-RAC1 ON BLOOD-BRAIN BARRIER STABILITY**  
**FOLLOWING RODENT NEONATAL HYPOXIA-ISCHEMIA.**

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The work presented in this chapter has been published.  
Acta Neurochir Suppl. 2016;121:263-7.

## **Abstract**

Osteopontin (OPN) is a neuroprotective molecule that is upregulated following rodent neonatal hypoxic-ischemic (nHI) brain injury. Because Rac1 is a regulator of blood-brain barrier (BBB) stability, we hypothesized a role for this in OPN signaling. nHI was induced by unilateral ligation of the right carotid artery followed by hypoxia (8 % oxygen for 2 h) in P10 Sprague-Dawley rat pups. Intranasal (iN) OPN was administered at 1 h post-nHI. Groups consisted of: (1) Sham, (2) Vehicle, (3) OPN, and (4) OPN + Rac1 inhibitor (NSC23766). Evans blue dye extravasation (BBB permeability) was quantified 24 h post-nHI, and brain edema at 48 h. Increased BBB permeability and brain edema following nHI was ameliorated in the OPN treatment group. However, those rat pups receiving OPN co-treatment with the Rac1 inhibitor experienced no improvement compared with vehicle. OPN protects the BBB following nHI, and this was reversed by Rac1 inhibitor (NSC23766).

## **Introduction**

Neonatal hypoxic-ischemic brain injury remains a leading cause of mortality and morbidity, affecting 2-4 of 1,000 full-term and nearly 60% of premature infants [1,2]. Among survivors, 20-40% develop significant neurological impairments, associated with life-long medical, social, and emotional difficulties [3]. Recent studies have demonstrated neuroprotective effects of intracerebroventricular (ICV) administration of recombinant osteopontin (rOPN) [4]. Previously, rOPN was shown to have reduced cell death, stabilized the blood-brain barrier (BBB), and improved neurological impairments in rodents subjected to brain injury [5].

In sum, osteopontin (OPN) is a neuroprotective molecule that is upregulated following rodent neonatal hypoxic-ischemic brain injury [5,6]. OPN, an extracellular matrix protein, stimulates cell surface integrin receptors, which induce the phosphorylation of focal adhesion kinase (FAK) [4,7,8] which induces Rac-1, a downstream target of FAK that has been shown to stabilize the BBB after HI insult [9]. Because Rac1 is a regulator of BBB stability [10-13], we hypothesized a role for this in OPN signaling.

## **Methods**

Neonatal hypoxia-ischemia was induced by unilateral ligation of the right carotid artery followed by hypoxia (8% oxygen for 2.5h) in P10 Sprague-Dawley rat pups. Intranasal (iN) OPN was administered at 1 h post-nHI. Groups consisted of: (1) Sham, (2) Vehicle, (3) OPN, and (4) OPN+Rac1 inhibitor (NSC23766). Evans blue dye extravasation (BBB permeability) was quantified 24 h post-nHI, brain edema at 48 h.

### *Animal Surgery*

After induction of anesthesia, the neck of the rats was prepared and draped using standard sterile techniques. Skin preparation consisted of alcohol swabbing of the neck. Next, a small midline neck incision on the anterior neck was made with a No. 11 blade surgical knife. This incision was approximately 3-5 mm in length. Using gentle blunt dissection, the right carotid artery was isolated and gently separated from surrounding structures, including the vagus nerve, trachea, and esophagus. The carotid artery was then ligated with 5-0 surgical suture. All bleeding was controlled with gentle pressure and electrocautery as needed. After the carotid artery was ligated, the surgical field was irrigated with several drops of saline, dried, and the skin closed with sutures. The entire surgical procedure took approximately 10 minutes. After the surgical procedure was completed, the rats were allowed to wake and recover for 1 h. This recovery occurred in a temperature-controlled incubator. Throughout the surgical and post-operative period, temperature was controlled with heating blankets and incubators. After the rats recovered, they were returned to the care of their mother. After the 1-h recovery period, rats were exposed to hypoxia using the standard published protocols of the Rice-Vannucci model, done previously [14]. Exposure to hypoxia involved placing the animals in a temperature-controlled chamber and then exposing them to 8% oxygen in balanced nitrogen gas for 2.5 h. This hypoxic treatment was rendered in a specially designed treatment chamber to prevent escape of the hypoxic gas mixture. Gas composition in the chamber was monitored continuously with a monitor that measures oxygen and carbon dioxide content. After hypoxic treatment all animals were monitored closely for any signs

of distress, failure to thrive, infection, or serious disability. Immediately after hypoxia, animals were returned to the care of their mother.

### ***ICV Injection***

Animals were anesthetized with isoflurane. A scalp incision was made on the skull on the surface and the bregma exposed. Infusion of Rac1 inhibitor (NSC23766) was performed using a 10- $\mu$ l syringe (Hamilton Co., Reno, NV, USA) at the location 1.5 mm posterior and 1.5 mm lateral to the bregma, and 1.7 mm deep to the skull surface at the contralateral hemisphere 24 h before HI. Control rats were injected with 2% DMSO (2 $\mu$ l/pup, diluted in saline; Sigma). The injection was completed in 5 min and the needle maintained in the injection position for an additional 2 min. Then the needle was removed slowly out of the brain and the wound sutured. After recovery from anesthesia, the pups were returned to their dams.

### ***Evans Blue***

The spectrophotometric measurement of Evans blue dye [15, 16] was initiated through intraperitoneal (IP) injection after nHI to measure BBB permeability. Under anesthesia, Evans blue (Sigma-Aldrich) in normal saline (2%, 4 ml/kg) was infused. For quantitative measurements, the brain hemispheres (hemorrhage) were homogenized in 3 ml of N, N-dimethylformamide (Sigma-Aldrich), incubated for 18 h at 55°C, and then centrifuged. Supernatants were analyzed at 620 nm by spectrophotometry, as routinely performed [17].

### ***Brain Edema***

Brain water content [15, 18-21] was measured using the wet-weight/dry-weight method [17]. Following sacrifice, the brains were removed and divided. Tissue weights were measured before and after drying for 24 h in a 100°C oven. Weights were measured using an analytical microbalance (model AE 100; Mettler Instrument Co., Columbus, OH, USA) capable of precision within 1.0 µg. Brain edema was tabulated as percentage: (wet weight – dry weight)/wet weight x 100.

### ***Statistical Analysis***

Significance was based on  $p < 0.05$ . Data were statistically analyzed using one-way analysis of variance (ANOVA), followed by Tukey post hoc test for significant analyses. Statistical analyses were performed using SigmaPlot version 10.0 for Windows.

### **Results**

A schematic of the proposed mechanism used as the basis of this preliminary work is outlined (Fig. 1). Experimental data show significantly increased BBB permeability (Fig. 2) and brain edema (Fig. 3) following nHI. These brain injuries were significantly improved in the OPN treatment group. However, the rat pups receiving OPN cotreatment with the Rac1 inhibitor experienced no improvement compared with vehicle (please see Fig. 2), mechanistically reversing neuroprotection.



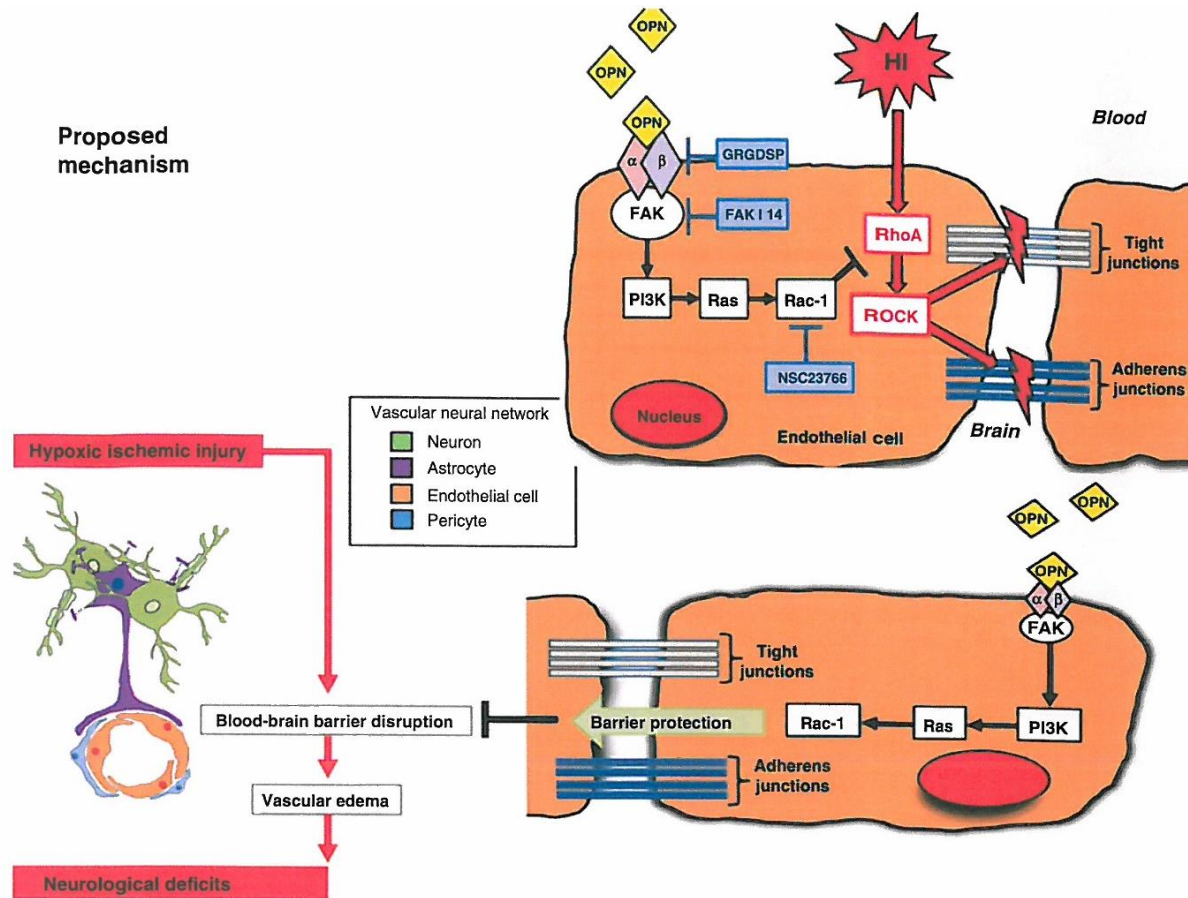


Figure 22: Schematic, showing proposed mechanisms involving this preliminary investigation, directives of further study.

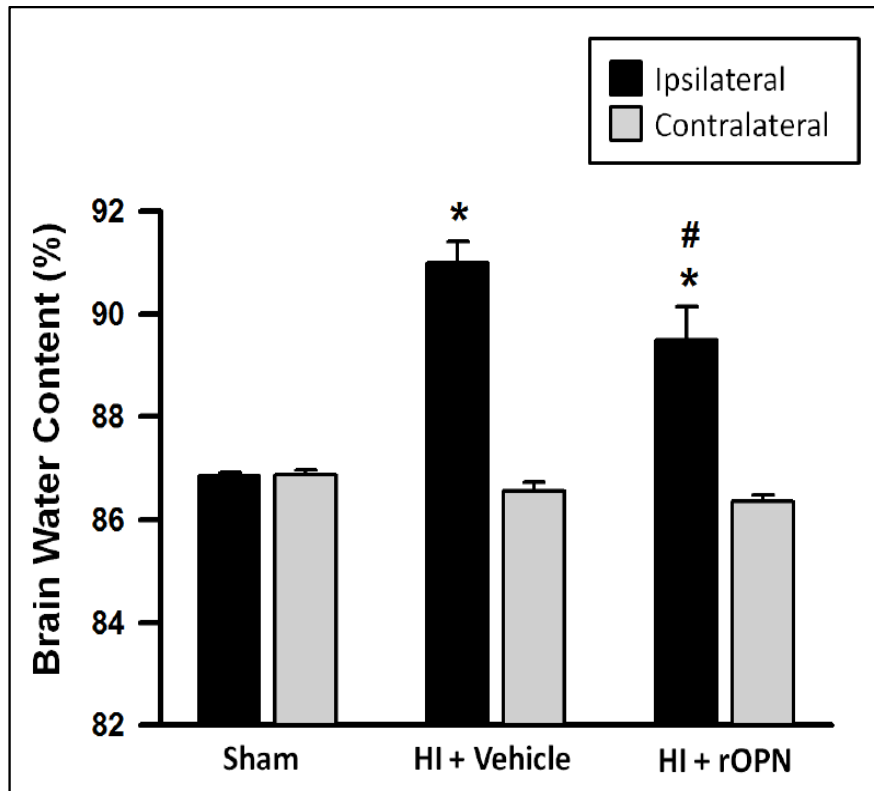


Figure 23: OPN reduced brain edema. To determine the effects of OPN on BBB permeability, brain water content, which is a measure of brain edema, was evaluated. Brain edema was significantly increased in the ipsilateral hemisphere following HI induction. OPN significantly reduced brain edema compared with vehicle group. The contralateral hemisphere showed no increase in edema (\* $p < 0.05$  vs. sham; # $p < 0.05$  vs. vehicle;  $N = 4/\text{group}$ ).

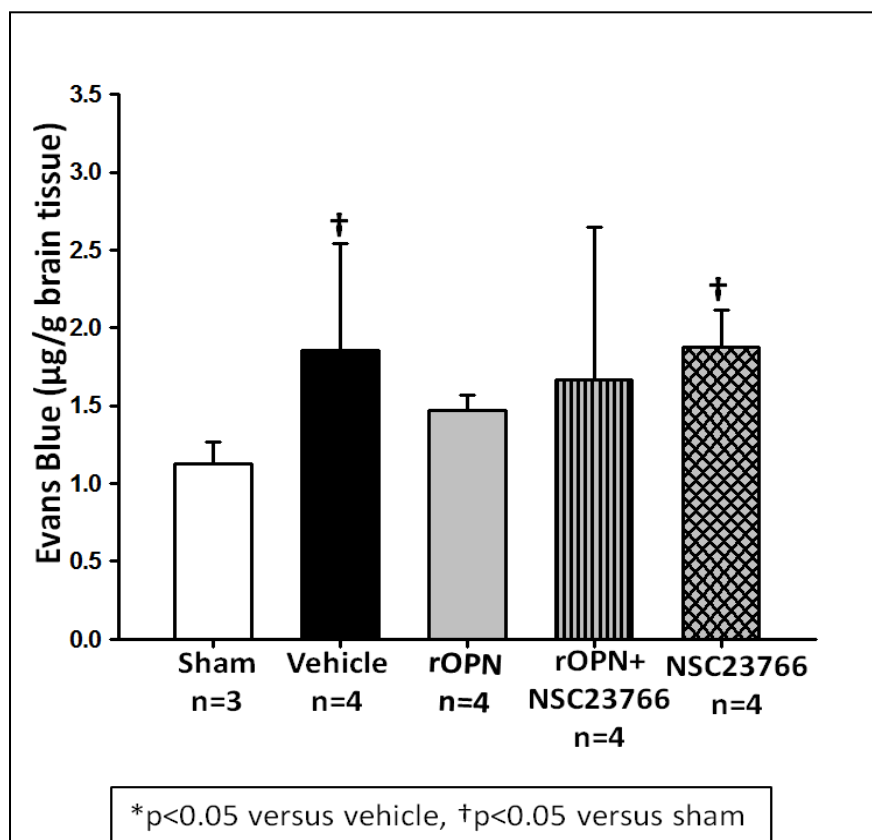


Figure 24: OPN reduced BBB permeability. To determine the effect of OPN on BBB permeability, Evans blue dye extravasation as a measure of BBB permeability was evaluated. OPN (5g) was administered following HI induction, and HI significantly increased Evans blue dye extravasation, as seen in the ipsilateral hemisphere, which was significantly attenuated by OPN treatment.

## **Conclusion**

Translational stroke studies, including animal modeling, are greatly needed to safely integrate basic preclinical scientific principles ahead of clinical application [22-26]. Numerous attempts to reduce HI-induced consequences have failed in the clinical setting. Therefore, it is imperative to create translatable studies that incorporate unique characteristics of the immature brain, such as its anatomical structure, physiological function, cellular composition, and its response to injury [14, 25, 27-36].

The specific objective of this study was to determine a mechanism by which rOPN affected BBB permeability and brain edema in a well-established animal model of neonatal HI. Our data demonstrate that administration of rOPN improves recovery after neonatal HI by stabilization of the BBB, possibly via integrin receptor signaling pathway. The long-term goal of this study is to provide a further basis for the clinical translation of rOPN as an effective therapeutic option with long-term benefits.

In summary, this study showed that OPN protected the BBB following nHI, and this was reversed by Rac1 inhibitor (NSC23766). The mechanisms mediating this neuroprotection warrant further study.

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## **CHAPTER SIX**

### **GENERAL DISCUSSION AND CONCLUSION**

#### **Summary of Key Findings**

In the previous chapters we were able to demonstrate that IFN $\beta$ , G-CSF, and OPN were able to confer neuroprotection after HIE [1, 2]. Also we reviewed and discussed some of the impediments to developing neuroprotective strategies and the future of the field [3].

Our findings showed that intranasal administration of IFN $\beta$  was able to reduce neuronal apoptosis by activating the Jak1/Stat3/Bcl-2 pathway 24 hours post injury. We determined that IFN $\beta$  treatment induces Stat3 activation and leads to increased expression of Bcl-2 leading to a decrease in cleaved caspase-3 expression and neuronal degeneration. IFN $\beta$  was also able to reduce brain infarction volumes, increase post-injury weight, and improve short-term neurobehavior. Also Stat3 inhibition showed a reversal in the protective effects of IFN $\beta$  treatment. Our results also demonstrated that administration of G-CSF was able to reduce neuroinflammation and BBB disruption via the G-CSFR/PI3K/Akt pathway activation and subsequent GSK-3 $\beta$  inactivation following HIE. Moreover, G-CSF was able to decrease the expression of pro-inflammatory (IKK $\beta$ , NF- $\kappa$ B, TNF- $\alpha$ , IL-1 $\beta$ , and IL-12) while also increasing the expression of IL-10, an anti-inflammatory cytokine [1]. We also found that OPN was able to improve brain edema and reduce blood-brain barrier permeability following HIE as well [2]. Our data suggests that OPN improves recovery and stabilizes the BBB after neonatal HIE via the integrin receptor signaling pathway, since OPN mediated protection was reversed when a RAC1 inhibitor was applied [2].



### **How Our Findings Advance the Field?**

Our scientific experiments and review article publications have been able to significantly contribute to the field of neonatal HIE. We were able to effectively discuss how to develop neuroprotective strategies and translation of experimental results in HIE, the many limitations and challenges to create appropriate study designs, drug administration, and usages in neonates. We also identified understudied targets after HIE, neuroprotective molecules, recent trials being conducted in the clinical setting, and future directions [3]. Also we explored and analyzed the current information concerning isoflurane as an neuroprotective agent after neonatal HIE [4]. Our study was also the first to explore the effects of IFN $\beta$  treatment after neonatal HIE, as well as the first to characterize that IFN $\beta$  reduces neuronal apoptosis via the Jak1/Stat3/Bcl-2 pathway after HIE. We also helped assist in the basic science research foundation of G-CSF and OPN, since we found that G-CSF treatment reduces BBB disruption via G-CSFR/PI3K/Akt/GSK-3 $\beta$  pathway and that rOPN stabilizes the BBB via the integrin receptor [1, 2]. Our findings in these areas have produced the need for future studies to further characterize and translate G-CSF and rOPN has possible clinical treatments. For example further studies to examining the role of G-CSF in reducing cerebral edema and the mechanisms by which G-CSF-mediated GSK-3 $\beta$  inactivation decreases VCAM and ICAM and increases the expression of claudins should be explored. Also the anti-apoptotic and anti-inflammatory properties and mechanisms of rOPN should also be explored in the future.

## **Current Limitations to Neuroprotective Strategies after HIE**

### ***Limitations to in Vitro and in Vivo***

The distinct mechanisms of injury-induced damage in immature brain is frequently studied, yet remains poorly understood, especially at early stages of development. Also, a thorough evaluation method of the developmental and behavioral sequelae after brain injury needs to be established [5].

In general, there are in vitro and in vivo models of experimental studies. In-vitro studies are relatively inexpensive and often precede a following in vivo analysis, yet it has its limitations of treating cells outside of their normal environment. Therefore, their results may differ from in vivo effects [6]. If in vivo tests can confirm in vitro results, the informative value of the results is more significant. In most cases, drug studies proven to be effective in vitro, show ineffective in animal models. The underlying reason may be drug delivery issues, toxicity, or other problems [7]. Even though several reviews demonstrated poorly predictive value of animal models on human outcomes, those experiments are mandatory especially in drug development before translation to clinical trials [8-10].

### ***Limitations to Animal Models***

Especially since HIE and neonatal stroke are very heterogeneous entities, therefore finding the ideal method to study this disorder remains a difficult task [11]. Ashwal and colleagues compared the HIE model by Rice-Vannucci and the neonatal stroke filament occlusion model using MRI. Obviously, the stroke model injury was defined on the ipsilateral middle cerebral artery territory, while more generalized and

greater cortical ischemia occurred with the Rice-Vannucci method, even involving the contralateral side in some cases [12].

Other new promising neonatal hypoxic-ischemic models have been introduced recently, like a perinatal global ischemic brain injury model in rats providing intrapartum hypoxia in rats [13]. Another perinatal method accomplished systemic hypoxic-ischemic brain injury by cutting off the placenta blood supply to the fetuses to mimic brain damage in early preterm newborns [14]. One model of postnatal permanent occlusion of the middle cerebral artery was described using direct electrocoagulation aiming for a highly reproducible, more consistent, and selective cortical infarction area [15].

The great variety of established models and promising new designs points out the importance of carefully choosing the appropriate model, especially for testing possible treatment strategies [16]. A few key points may be that the timing and nature of the induced ischemic injuries vary tremendously in the different models. Additionally, the translation from rodent studies to human trials are discussed broadly, because of diverse and in some extent lacking neurological responses to sensorimotor cortex lesions when compared to humans. This may demand non-human primate experiments for more neurological similarity [17]. Thus, only one perinatal hypoxic-ischemic brain injury model in primates has been developed so far, showing similar anatomical and cellular pathology in cortex development, as in post-ischemic observations in newborns [18]. Although, biologically proximity to humans seems to be important in animal studies, over 95% of experiments in general were performed in rats and mice [19, 20]. Other points to consider are the inevitable delays between symptom onset and start of treatment [21, 22]

or the outcome assessment within days in animal models in contrast to after months in patients [23].

Although much progress in experimental development and technology has been made in the past few decades, the issue of discovering disease models, that can mimic human pathophysiology sufficiently, remains an ongoing challenge [20, 24, 25].

### ***Limitations to Central Nervous System Drug Delivery***

Treatment strategies for central nervous system (CNS) diseases are challenged by the drug delivery mechanisms for crossing the BBB [26]. The underlying reason, also considered the bottleneck in CNS drug development, is in most cases since insufficient attention to the prediction and assessment of the compounds ability to cross the BBB [27]. Without solving the BBB problem, CNS drugs are limited to lipid-soluble and low molecular weight (less than 400 Daltons) compounds. Progress in molecular neuroscience might therefore lead to more availability of effective CNS therapeutics [28]. This demands research focusing on signaling pathways and trafficking mechanisms, as involved brain transporters at the BBB and brain-cerebrospinal fluid-barrier [29].

In a recent publication, rapid and transient BBB disruptions were described after HIE in neonatal mice associated with alteration of tight-junction proteins. This response is often followed by activation of tightening compensatory mechanisms. More understanding of this phenomenon might provide an opportunity window for compounds to access the brain tissue easier after HIE [30].

### ***Limitations to Neonatal Drugs and Dosage***

Additionally, in cases of neonatal HIE the clinical translation is more difficult when compared to adult drug administrations, since most of the pharmacological developments are approved only for adults and the regulations for pediatric and neonatal use are highly restricted. Due to the small size and huge variability of pediatric patients, neonatal pharmacotherapies are limited to mostly off label usage of compounds developed for adults to extrapolate dosages [31-33]. Another issue besides the small number yet huge variety of patients challenging clinical trials and regulations are possible side effects, as in adverse drug reactions in the individualized neonatal treatment strategies [34].

Progressive research in neonates might improve and optimize future age-appropriate treatment demanding tailored, personalized clinical pharmacological and physiological understanding [35-38]. Further research topics involve drug excipients for analyzing and initiating the safety and toxicity for pediatrics (STEP) database and the European Study of Neonatal Excipient Exposure (ESNEE) initiative [39-41].

### **Conclusion and Future Perspectives**

Although there have been significant strides in the basic sciences to create novel neuroprotective and therapeutic strategies to combat HIE, there is still much more research needed to be conducted in order to translate potential therapies. Some gaps in our knowledge concerning the pathophysiology and the timing of important endogenous neuroprotective and neuroregenerative mechanisms still exist. In order to make basic science results more clinically relevant and translational, combinational therapies with

hypothermia should be considered and studied. There is also a need for more biomarker studies that can be used along with the brain imaging, and long-term neuro-assessments [42, 43]. Thus, the field of neonatal HIE has many promising avenues and possibilities in the realm of translational research.

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